DEVELOPMENT OF MOLECULAR TOOLS FOR THE CHARACTERISATION OF FOOT-AND-MOUTH DISEASE VIRUS STRAINS CIRCULATING IN SOUTHEAST ASIA

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy by

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October 2010

Abstract

Foot-and-mouth disease is an acute and contagious vesicular disease affecting wild or domesticated cloven hoofed animals. The disease is caused by Foot-and-mouth disease virus (FMDV) of *Aphthovirus* genus within *Picornaviridae* family. FMDV exists as seven distinct serotypes; O, A, C, Asia 1, SAT (Southern Africa Territories) 1, SAT 2 and SAT 3, each with multiple subtypes which are not equally distributed around the world. In this study, VPI analysis of clinical samples cattle and pigs received from Southeast Asian countries namely Malaysia, Thailand, Lao PDR, Myanmar, Vietnam and Cambodia between 2000 to 2009 revealed seven FMDV lineages circulating in the region. The lineages were O/SEA/Mya-98, O/SEA/Cam-94, O/ME-SA/PanAsia, O/ME-SA/PanAsia-2, O/CATHAY, A/ASIA, and Asia 1. Some of these lineages were highly prevalent such as O/SEA/Mya98 and serotype A, while O/ME-SA/PanAsia-2 was unique to Malaysia within Southeast Asia. Phylogenetic analysis of VP1 showed two distinct patterns of genetic relationships between viruses from Malaysia and the other neighbouring countries. These data suggest that viruses of O/SEA/Mya-98 and serotype A lineage were repeatedly introduced into the country on a yearly basis. In contrast, viruses belonging to O/ME-SA/PanAsia-2 sublineage were maintained in the country to cause outbreaks in following continuous years. However, phylogenetic analysis cannot conclude the link between Malaysian isolates with those from neighbouring countries. Since Malaysia is a major beef and animal importer, the sources of these FMDVs were most likely introduced via imports of infected animals or contaminated animal products into Malaysia. In order to improve the resolution, by which the molecular epidemiology of FMDV in the region can be studied, RT-PCR protocols to amplify complete genome sequences of each lineage were developed. The protocols utilised a single set of universal primers for NSP region and specific primers sets for the capsid region. Complete genomes from representative isolates from the seven virus lineages were generated from Malaysian samples. Basic characteristic of genomes generated with this protocol were consistent with previous published sequences such as deletions at the 3A region for O/CATHAY and O/SEA/Cam-94 lineage. In addition, preliminary analysis of O/ME-SA/PanAsia-2 sequence showed numerous nucleotide substitutions observed at the 5'UTR and L^{pro} region of genome to that of O/ME-SA/PanAsia which indicated a possible event of recombination. The O/SEA/Mya-98 protocol was proven to be robust when it was utilised to generate two complete genome sequences of O/SEA/Mya-98 viruses from outbreaks in East Asian countries, in Hong Kong SAR and Republic of Korea that have occurred during 2010. Analysis of the Hong Kong genome sequence revealed a block deletion of 70 nucleotides located within the S-fragment. Subsequent analysis showed that this feature was shared with other viruses with related VP1 sequences. The methods developed in this study provide a basis for a more comprehensive understanding of the diversity and evolution of FMDV that will hopefully develop improved epidemiological and diagnostic tools that will contribute to the existing disease control programmes in the region.

Acknowledgement

First and foremost, praise to Allah the Al-Mighty who has given me the strength and health to finish this study. This thesis would not have been possible without the support an encouragement, both scientific and personal of many great people. I am deeply indebted to my supervisors; Don, Alan and Nick who always believe in me from the beginning and always been very supportive and encouraging. No words can describe how grateful I am to have you all as my mentor. In the lab without help and assistance from Jemma, Muge and Caroline, I would have not been able to finish my study. Thank you to you and all the others in the lab; Anna, Jo, Claudia and Valerie who have made my two years work in the lab an enjoyable time. I'd also like to extend my gratitude to Houssam and Fauziah for the friendship and guidance. There are also people from outside the lab who have been supported me technically, emotionally and socially. Special thanks to Department of Public Services and Department of Veterinary Services Malaysia who have given me the invaluable opportunity to study in the UK. My love and gratitude goes to my dear husband, Huzaymi for his unconditional love and endless patience throughout the wonderful and painful moments. To my two lovely tots, Muhammad and Nurul who always showered me with their hugs and kisses after a long day at work, their love makes me even stronger each day. I would have not succeeded without continuous pray and love from Mak, Abah, Akak, Aman and Ik. Finally, I must also thank all Malaysian friends in University of Surrey who have lightened up our life in the UK, without whom our life would not be fun.

Table of contents

List of Figures

List of tables

Abbreviations

bp	base pairs
BTy	bovine thyroid
cDNA	complementary DNA
DEFRA	Department for environment, farming and rural affairs
DNA	deoxy-ribonucleic acid
dNTP	deoxy-nucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FAO	Food and agriculture organisation
FMD	foot-and-mouth disease
FMDV	foot-and-mouth disease virus
FS	first strand
IRES	internal ribosome entry site
MCC	maximum crade credibility
LAMP	Loop-mediated isothermal amplification
MCMC	Markov Chain Monte Carlo
ME-SA	Middle East-South Asia
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
ml	millilitre
mM	millimolar
NF	nuclease free
NJ	Neighbor-joining
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RS	renal swine
RT-PCR	reverse transcriptase - polymerase chain reaction
SAT	South African Territories
SEA	Southeast Asia
SEAFMD	Southeast Asia foot-and-mouth disease
UTR	untranslated region
v	volts
VP1-4	viral protein 1-4
WRL	World Reference Laboratory
µl	mikrolitre
µM	mikromolar

Chapter 1

Introduction to foot-and-mouth disease

1.1 Foot-and-mouth disease

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animal such as cattle, buffalo, pigs and small ruminants (Alexandersen et al., 2003). It is often considered the most important animal disease as a consequence of its impact upon rural economies and international trade. It was listed by the OIE in their notifiable disease list due to its ability of rapidly spread regionally and internationally. The disease is endemic in Asia, Africa, South America and Middle East (Gleeson et al., 2003; Kitching, 1998; Knowles and Samuel, 2003). Some countries within these regions are free from the disease where occasionally FMDV can cause sporadic outbreaks.

1.2 Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) was the first animal pathogen identified as a virus (Brown, 2003; Loeffler and Frosch, 1898). FMDV is classified in the *Aphthovirus* genus and belongs to the *Picornaviridae* family. FMDV is a small virus sized about 30nm and has a positive-sense single strand RNA genome of about 8500 nt in length. The virus is sensitive to an acidic and alkaline environment, high temperature (>50C) and UV light (Forss et al., 1984). However under certain circumstances and temperate conditions it can be sustained in the environment such as in contaminated fodder for up to one month.

1.3 FMDV serotypes

The virus is grouped into seven serotypes; O, A, C, Asia 1, SAT 1 (Southern African territories), SAT 2 and SAT 3 based on the nucleotide sequence of the VP1 (1D) structural protein of the capsid (Knowles and Samuel, 2003; Marquardt and Adam, 1990). The VP1 gene is important due to its role for antigenicity, immunity

and serotype specificity (Jackson et al., 2003). Within serotypes there are multiple topotypes that are usually related to geographical region of the disease occurrence or subtype. Many studies have been undertaken previously to characterise and group viruses of each serotype to better understand the geographical movement of FMDV and identify the source of new outbreaks.

A study on genetic diversity of FMDV serotype O has been carried out by (Samuel and Knowles, 2001) who classified this serotype into eight topotypes using a value of 15% nucleotide differences to discriminate between topotypes. More recently this serotype has been grouped into 11 topotypes namely Middle East-South Asian (ME-SA), Southeast Asia (SEA), Europe-South America (Euro-SA), West Africa (WA), East Africa 1 (EA-1), EA-2, EA-3, EA-4, CATHAY, Indonesia-1 (ISA-1) and Indonesia-2 (ISA-2) (Knowles et al., 2008)

Serotype A viruses are considered to be extremely antigenically and genetically diverse such that in early 1970s, 32 subtypes had been described (Pereira, 1977). A number of studies have shown that viruses of this serotype fall into numerous genetic groups based on complete or partial VP1 sequencing. Tentatively FMDV serotype A can be grouped into three major geographical topotypes; i) Euro-SA ii) Asia and iii) Africa (Knowles and Samuel, 2003).

Serotype Asia 1 is unique to Asia region where it causes only a small proportion of FMD cases. Even though a wide variation were observed in VP1 nucleotide sequences, it was not sufficient to classify Asia 1 viruses into more than one topotype (Knowles and Samuel, 2003). A recent study on viruses belonging to serotype Asia 1 from outbreak during 2003 to 2007 in Asian countries has characterised these viruses into six different groups, I to VI (Valarcher et al., 2009).

1.4 FMDV genome structure

The genome of FMDV is over 8000 bases in length and encapsidated in an icosahedral structure of outer protein (Jackson et al., 2003; Mason et al., 2003a). The FMDV ORF (open reading frame) can be divided into four regions based on the presence of cleavage sites; L^{pro}, structural protein (P1) and non-structural proteins (P2 and P3) (**Figure 1.1**) (Robertson et al., 1985; Rueckert and Wimmer, 1984). The L^{pro} contains two in-frame AUG initiation codons that encode two L proteins, Lab and Lb. The P1 region of the genome encodes the four structural proteins, 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) that make up the outer coat of virus. The outer protein coat of FMD virus, also known as the capsid is made up of 60 copies each of the four structural proteins. VP4 protein is internally in contact with RNA while the other three proteins are on the surface. These four proteins assemble to form a protein sub-unit or protomer and later five protomers join to form a pentamer. Twelve pentamers join up enclosing a strand of RNA to create a virus particle called provirion. The P2 region encodes three non-structural (NS) proteins namely 2A, 2B and 2C and the P3 region encodes another three NS proteins, 3A, $3C^{pro}$ and $3D^{pol}$ protein. In addition, FMDV has two untranslated regions (UTRs) at both ends of the genome. The 5'UTR region consists of the S-fragment, poly C, pseudoknot, cre structure and IRES (internal ribosome entry site) which is over 1300 bases (Forss et al., 1984). At the other end of the FMDV genome is the 3'UTR region with a poly-A tract (Dorsch-Hasler et al., 1975).

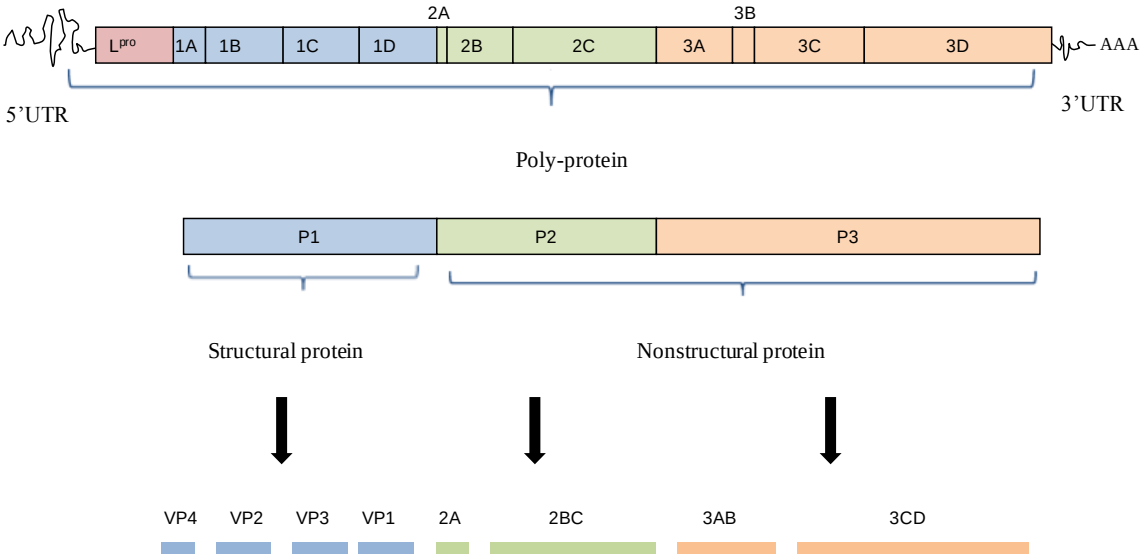

Figure 1.1: Schematic diagram of FMDV genome extracted from (Mason et al., 2003a). Nomenclature is based on (Rueckert and Wimmer, 1984). Also shown are the cleavage site and the products.

1.5 Pathogenesis

1.5.1 Routes of infection

Direct contact between infected and susceptible animals is the most common route of infection. The virus can enter the susceptible animal through cuts or abrasions or in the form of a droplet deposited into the respiratory tract. This mode of infection is facilitated by close physical contact between naïve and infected animals (Alexandersen et al., 2002a). This becomes a major problem in countries such as those in the Southeast Asia (SEA) region where the farmers practise free grazing. In this scheme, animals of various farmers are let out into grazing fields thereby increasing the likelihood of virus transmission between holdings if one of the animals was infected. Another important route to consider is by oral infection, particularly where swill feeding is practised (such as in part of SEA) since this route

can attribute to disease spread. The outbreaks in Japan, South Africa and the UK in year 2000 and 2001 respectively were suggested to be related to feeding pigs with contaminated waste food (Knowles et al., 2001b). The chances to get infected could be more if animals have abrasion around the mouth which facilitates the virus entry (Donaldson et al., 1987). Contact with virus from infected animals can also occur indirectly via fomites such as contaminated bedding, vehicles, personnel and farming equipment. Another route that can contribute to field spread is airborne transmission when under certain climatic condition virus droplets containing FMDV can be transmitted over longer distance (Alexandersen and Donaldson, 2002).

1.5.2 Incubation period

The incubation period for FMD depends on the strain and dose of virus, the route of transmission, the animal species, the susceptibility of animal host as well as the husbandry management. Spread within a herd takes between 2 to 6 days although under certain circumstances it can be as short as 1 day. Transmission between farms has a longer incubation period but once the amount of virus in the infected farm increases, the incubation period reduces (Kitching, 2002).

1.5.3 Sites of infection

The pharyngeal area is thought to be an important initial site of infection unless the virus has gained entry through cuts or abrasions. The pharyngeal area is comprised of dorsal soft palate, roof of pharynx and the part of the tonsil which lined with stratified squamous non-cornified epithelial cells. The non-cornified feature provides live cells for virus replication and it also helps in maintaining persistent infection in carrier animals. Virus will stay in the pharynx and can be demonstrated

for 1 to 3 days before it enters the blood stream via regional lymph nodes to cause viraemia (Alexandersen et al., 2002c).

1.5.4 Clinical signs

FMD is characterised by an acute febrile condition and the formation of vesicles in the mouth and on the feet. The resultant pain leads to weakness and inappetance. Following a period of initial pyrexia of 40ºC which can last for one to two days, a variable number of vesicles develop on the tongue, hard palate, dental pad, lips, gum, muzzle, coronary band, interdigital spaces, teat and snout in pigs (Alexandersen et al., 2003). These signs sometimes go unnoticed in real field conditions such as free grazing or semi intensive husbandry systems. Eventually within 24 hours the vesicle erupts leaving a fresh, raw ulcer lesion. At this stage, the animal may show signs of excessive frothy salivation with protruding tongue or frequent smacking of the lips. The vesicles in the feet remain intact for two to three days before rupturing depending on the flooring accommodation. The open lesions in the mouth, on the feet, teat and snout predispose the animal to secondary infections which may lead to chronic lameness or mastitis. Healing of the mouth lesions starts with fibrin covering the raw lesion and eventually ulcers appear as areas of pink fibrous tissue without normal tongue papillae. Infection in young calves can be fatal because the predilection of the virus to invade and destroy cells of the developing heart muscle (Kitching, 2002). Differential diagnoses of these clinical signs apart from FMD are vesicular stomatitis, swine vesicular disease and vesicular exanthema of pigs.

1.5.5 Excretion of virus by infected animals

All secretions and excretions become infectious during the course of the disease and some contain significant titres even before the development of clinical sign. Large amounts of virus are excreted in vesicular fluid, desquamated vesicular epithelium as well as saliva (Scott et al., 1966). Virus is also excreted in milk and semen from shortly before clinical signs appear and throughout the clinical phase (Burrows, 1968). There is also virus excretion in faeces but to a much lesser extent (Parker, 1971). A sharp decline in the excretion and load of FMDV is observed around day 4 to 5 of clinical disease, that is coincides with the development of significant detectable circulating antibodies to the virus (Alexandersen et al., 2003).

1.5.6 Persistent infection

Persistent infection or also called carrier state is characterised by asymptomatic low-level excretion of the virus from the oropharynx of animals for periods that are species and virus strain-dependent (Salt, 1993). This situation can be demonstrated in a proportion of ruminant animals convalescent from infection as well as in vaccinated ruminants following exposure to infection (Alexandersen et al., 2002b). The earlier study has defined the period of persistent infection for cattle, sheep and goat was a minimum of 28 days after infection (Sutmoller and Gaggero, 1965). There were many opinions on carrier state of pigs. Earlier study has showed that pigs can become carriers (Mezencio et al., 1999) and supported by a recent study when virus was isolated at 26 days post contact (Carrillo et al., 2007). However another study showed otherwise with no detectable virus at three weeks post infection (Alexandersen et al., 2003). For other species such as African buffalo (Syncerus caffer), it can carry the virus for up to 5 years (Condy et al., 1985). As for wildlife species such as deer and impala, an important role as carriers is unlikely

(Bastos et al., 2000). Even though the risk of transmission from carrier animals appears to be very low and requires certain, as yet undefined, trigger factors, it cannot be totally excluded due to its major consequences for the livestock international trade.

1.6 Laboratory diagnosis

Accurate diagnosis of FMD must be carried out at specialised laboratories. Complement fixation test was the earliest laboratory test used to diagnose FMD. This test was later replaced by ELISA due to its sensitivity and specificity. This method is able to confirm the clinical diagnosis and identify the FMDV serotypes (Ferris et al., 1988). Molecular methods, such as conventional RT-PCR assays have been developed and also can provide serotype-specific results (Reid et al., 1999). However the number of samples that can be analysed simultaneously with this technique is limited and this approach may not be able to cope with samples that might be received during an epidemic. Therefore real time RT-PCR was developed and has been shown to have high sensitivity and specificity for the detection of FMDV genomes of all seven serotypes (Reid et al., 2002). This assay has been used on a large number of tissues samples, serum samples, swab samples and tissue culture supernatants. Another antigen detection method that has been developed is the "lateral flow device" (LFD) which has been evaluated and shown to be pan-reactive to all FMDV serotype except for serotype SAT 2. Since this technique was easy and rapid, it has the potential to be used for pen-side diagnosis for FMD suspected outbreak (Ferris et al., 2009).

1.7 Foot-and-mouth disease in Southeast Asia (SEA)

Foot-and-mouth disease has been known to be endemic in most SEA countries namely Thailand, Cambodia, Lao People's Democratic Republic (PDR), Peninsular Malaysia, Myanmar and Vietnam as shown in **Figure 1.2** (Gleeson et al., 2003). The other countries, Brunei, Indonesia, East Malaysia and Singapore are recognised internationally free of the disease without vaccination. Indonesia has sustained the disease freedom status after eradicating the disease during the 1980s. For the Philippines, while large parts of the archipelago (Mindanao, Visaya, Palawan and Masbate), the virus still circulated in swine population in the south of Luzon island. This resulted in complication of FMD eradication process from the country (Rweyemamu et al., 2008). In the region, disease spread was always related to animal movement within the country or across international borders either for the plantation of rice paddies as in early 1990 or more recently for meat supply (Rweyemamu et al., 2008).

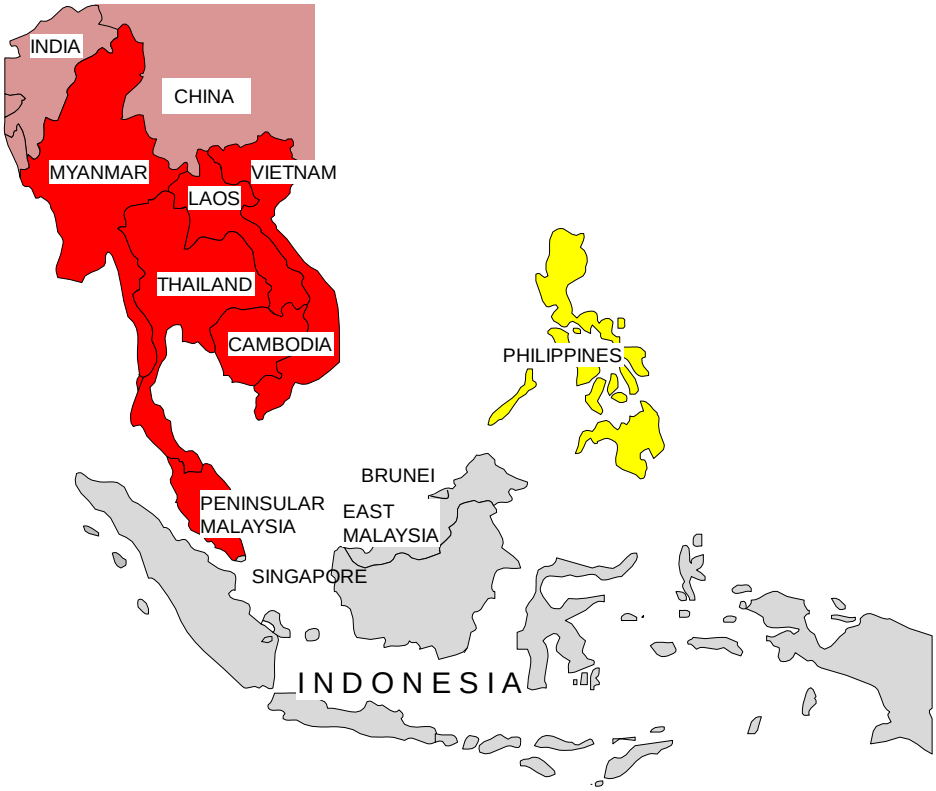

Figure 1.2: Map of countries in Southeast Asia (SEA) region. The countries that are red in colour are the FMD endemic countries in the region, while those in brown are FMD endemic neighbouring countries. The countries in grey are the SEA countries which are free of FMD without vaccination. While the yellow labelled country (Philippines) is sporadic for FMD and undergoing a roadmap towards disease eradication.

1.7.1 Serotype O

There are three distinct topotypes of serotype O occurring in this region namely SEA topotype, CATHAY topotype and PanAsia strain which derived from the ME-SA topotype (Knowles and Samuel, 2003; Knowles et al., 2005).

1.7.1.1 SEA topotype

Virus belonging to the SEA topotype has predominated in most of the FMD-endemic countries of mainland of the SEA region. The SEA topotype appears to have evolved independently from those in the rest of Asia (Samuel and Knowles, 2001). It was reported that until 1999, only viruses belonging to this topotype were

seen in Southeast Asia countries before the incursion of new virus lineage (PanAsia virus) was reported (Knowles et al., 2000). Earlier, there are two main sub-groups within SEA topotype; Mya-98 lineage which consists of viruses from Malaysia and Myanmar; and Cam-94 which consists of viruses from the Indo-China region (Laos, Cambodia and Vietnam). Viruses from Thailand fall into both groups. It was suggested that this grouping reflects the animal movement patterns in the region (Gleeson et al., 2003). However, the Mya-98 lineage viruses has been reported in Vietnam (Le et al., 2010b) and more recently has caused outbreaks beyond this region, in the East Asia countries (Paton et al., 2010).

1.7.1.2 ME-SA topotype

The first incursion of the PanAsia strain of ME-SA topotype into Southeast Asia was reported in 1999 in Vietnam, Thailand and Laos. By the year 2000, this virus had made its way into Cambodia and Malaysia (Knowles et al., 2005). In light of its persistence, it was suggested the PanAsia lineage could displace the domination of the SEA topotype in the ruminant population (Gleeson, 2002). It remains to be seen if this new virus strain will become endemic in the region as it has in the Middle East (Samuel et al., 1997). Phylogenetic analysis has been carried out to characterise the genetic sub lineages of the ME-SA topotype (Knowles et al., 2005). Evidence points to India as the origin of these viruses. The viruses belonging to this lineage were presumed to spread to Middle East in 1998 affecting Bahrain, UAE, Iran, Saudi Arabia and Yemen.

1.7.1.3 CATHAY topotype

This topotype contains viruses which were previously found in Hong Kong and China. The viruses are highly adapted to pigs only causing outbreaks in this species (Samuel and Knowles, 2001). The porcinophilic property of this virus was a

result of a genome mutation in the 3A gene (Knowles et al., 2001a). In Southeast Asia this topotype has only been reported the in Philippines (precisely on Luzon Island) and Vietnam (Gleeson, 2002). In Vietnam, this topotype was initially detected in 1997 and later continued to cause outbreaks until 2004 (Knowles et al., 2005). The same virus has caused an epidemic in Taiwan in 1997 which devastated the pig industry resulting in losses in excess of more than USD100 million (Yang et al., 1999).

1.7.2 Serotype A

Serotype A is known to be antigenically variable and it has been recognised that this serotype has the potential to generate variants that may lead to vaccination failure. Earlier, in the Southeast Asia region, only Malaysia and Thailand were considered endemic with this serotype (Gleeson et al., 2003). Until relatively recently, viruses belonging to this serotype had never been reported in the Indochina region (Laos, Cambodia and Vietnam) although vaccination against this serotype was practised in Vietnam in early 1990s (Gleeson, 2002). However, recently there were outbreaks caused by serotype A in Laos and Vietnam (Khounsy et al., 2008; Le et al., 2010a). While Myanmar is an important country from where FMDV can potentially spread, it is interesting that type A virus has never been reported there although there was serological evidence of prior infection by this serotype in animals entering Thailand from Myanmar (Gleeson, 2002).

1.7.3 Serotype Asia 1

Asia 1 is a unique FMDV serotype due to the restricted distribution of these viruses primarily to the Asian continent. Serotype Asia 1 virus has spread periodically into the Middle East and occasionally to Europe but has never been

reported from Africa and the Americas. Even in FMD endemic areas, this serotype only causes a small proportion of FMDV outbreaks compared to serotype O and A. In Southeast Asia, outbreaks due to serotype Asia 1 have been reported only sporadically in the past 10 years (Valarcher et al., 2009). Retrospectively, it is important to note that between 1994 to 2001 this serotype was reported every year in Myanmar and most years in Thailand. In 1992 and 1997, it became the predominant serotype causing outbreaks in Thailand (Gleeson et al., 2003).

1.8 Disease control for FMD in SEA

FMD control received a great deal of attention in the countries of Southeast Asia since more than a decade ago when individual national control programs have been instigated at considerable expense. However these existing national programs were not sufficient especially in the mainland countries which share land borders. In the endemic situation, movement of infected animals within countries or across the international boundaries can readily lead to outbreaks in other neighbouring areas. Therefore, there was a need to form a regional coordination disease control program within the region. The Southeast Asia Foot-and-Mouth Disease Campaign (SEAFMD) was launched in 1997 (details available at http://www.seafmd-rcu.oie.int/index.php). It is coordinated by OIE with collaboration from international bodies as well as animal health authorities of member countries. The member countries of the campaign are Thailand, Cambodia, Lao People's Democratic Republic (PDR), Malaysia, Myanmar, Vietnam and the Philippines (Gleeson and Ozawa, 2002). SEAFMD 2020 has generated a roadmap and a strategic direction for achieving freedom from FMD with vaccination by 2020 in Southeast Asia (Murray, 2006). A number of challenges will be faced by the SEAFMD campaign to achieve these objectives (Gleeson and Ozawa, 2002; Makin, 2007). These include lack of

funding, traditional animal movement across borders, poor legislative control in most countries, underdeveloped veterinary professions as well as poverty. Following is a brief discussion of the important elements that impact upon disease control in the region:

1.8.1 Socioeconomic and political issue

(1) Attitudes of livestock holder/ farmers

The livestock production sector in Southeast Asia is often based on small scale farming to fit a variety of purposes. The animals could be used for family consumption, draught power or as financial resource. In these circumstances, the animal owners view that FMD is not a killer disease and that the infected animals will recover. This results in a general lack of enthusiasm for disease control programs. Furthermore, there is no incentive for farmers to eradicate the disease such as compensation for culling infected animals as is present in Europe countries.

(2) Attitudes of animal traders'

There is a significant industry that surrounds illegal animal movement activity across international borders, in which animal traders play an important role. It will be a challenge to any disease control program to influence the attitudes that accompany these smuggling and illegal slaughtering activities.

(3) Veterinary infrastructure and institutional development

The competence and resources of veterinary services in the different member countries vary. Generally there is a lack of well trained veterinarians with a basic disease control principles. Veterinary infrastructure must be strengthened in order to address these two factors.

(4) Animal movement

Recent changes in the political and economic growth have resulted in increased movement of livestock across international borders in the area. The international animal movement within this region is summarised in the **Figure 1.3**.

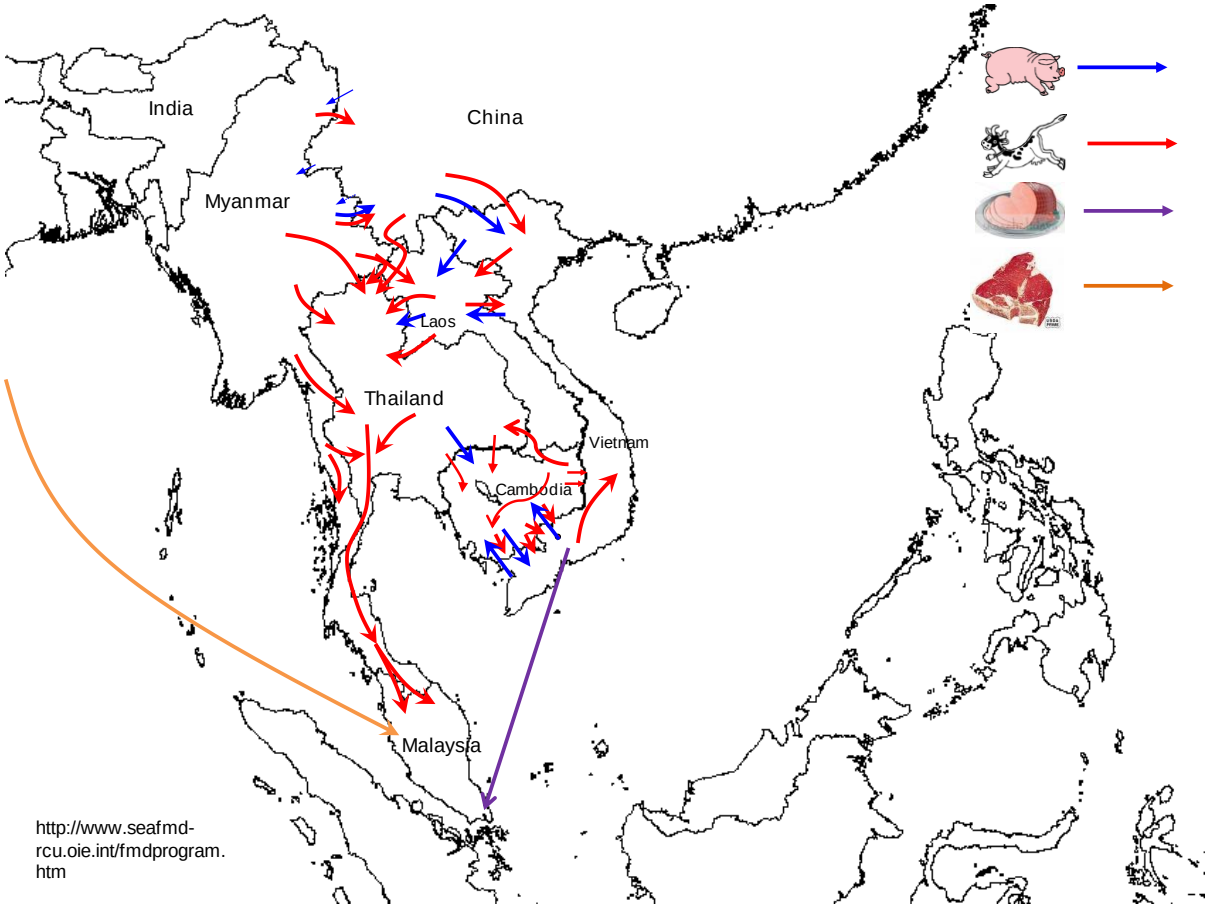

Figure 1.3: Map showing movement pattern of animal and animal product in Southeast Asia. Map was extracted from (Gleeson and Ozawa, 2002) with additional information from Disease Control Unit of Department of Veterinary Services, Malaysia. The red and blue arrows indicate the movement of live cattle and pig respectively. The orange and purple arrows indicate the movement of frozen beef and frozen suckling pigs respectively.

1.8.2 Technical issues

(1) Diagnostic test

The diagnostic capability is variable across member countries of the SEAFMD campaign. Training on diagnostic tests and financial support for equipment will be one aspect that needs to be emphasised in the SEAFMD campaign.

(2) Vaccines

In order to achieve the FMD freedom with vaccination objectives of the SEAFMD campaign by 2020, vaccine production must be available to prevent outbreaks in the field. Currently only Myanmar and Thailand have a national capacity to produce vaccine but these plants do not produce sufficient volume to meet local demands. Importing vaccine into the region will be more costly due to currency crisis or exchange rates. Therefore, there could be an opportunity to expand local production and possibly for export as well.

1.9 Objectives of my study

Malaysia has experienced FMD outbreaks probably as early as 1900 but not until 1935 were the first FMD outbreaks recorded (Babjee, 1980; Wallace, 1935). These occurred in the northern part of the country and since then, FMD has regularly been reported in livestock. Until recently, disease was diagnosed mainly based on clinical signs. In 1973, the first sample was sent to IAH, Pirbright for laboratory analysis and since then until today this collaboration continues to assist in sample analysis. The limitation of methods, instruments and expertise are the main constraint for Malaysia and other Southeast Asia countries to further analyse samples from outbreaks. Molecular analyses have been successfully used in other countries as a tool to understand the virus epidemiology at a local level, and this information later

contributes to improved disease control systems. Therefore three main objectives were outlined for this research:

1. Characterise lineages of FMDV that are circulating in SEA (Chapter 2).

To collate and critically review the molecular data that is available for FMDV strains from SEA. If possible, during the course of this project additional material (clinical material or virus isolates) will be collected and sent to Pirbright for VP1 sequencing.

2. Generate a protocol for high resolution molecular epidemiology (Chapter 3 and 4).

Complete-genome sequences of FMDV have been used recently as a tool to reconstruct the transmission pathways of the virus during outbreaks in the United Kingdom (Cottam et al., 2008). As an initial step to being able to conduct these studies in an FMD-endemic region, this project will develop protocols that will allow the amplification and sequencing of full-genome sequence for the predominant lineages of FMDV that are circulating in SEA, namely serotype O (all three topotypes), serotype A and serotype Asia 1.

3. Development of tailored diagnostic RT-PCR assay for SEA.

Rapid detection and characterization of FMD viruses causing outbreaks is important for disease monitoring and also plays an important role in the efficient use of vaccine to control the spread of FMD. Rapid real-time RT-PCR assays have been developed in IAH and elsewhere for the detection of FMD in a wide range of clinical sample (Oleksiewicz et al., 2001; Reid et al., 2002). However, these assays are typically pan-serotypic (detecting all seven FMDV serotypes), and are therefore unable to distinguish between different viral lineages. Using the sequence data obtained from objectives 1 and 2 of this project, new real time RT-PCRs will be designed for the detection of the major FMDV lineages in SEA. These assays will allow diagnostic

laboratories in the region to rapidly test clinical samples and discriminate the particular lineage of FMDV causing the outbreaks in the field.

It is hoped that this study will help not only Malaysia but also other SEA countries to develop approaches to improve control of the disease in the region.

Chapter 2

Phylogeography of foot-and-mouth disease virus types O and A in Malaysia and surrounding countries

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Work described in this chapter includes archival data from WRL sequence database (111 sequences).

This work has been accepted for publication in: *Infection, Genetics and Evolution*

Abstract

Foot-and-mouth disease (FMD) is endemic in the countries of mainland Southeast Asia where it represents a major obstacle to the development of productive animal industries. The aim of this study was to use genetic data to determine the distribution of FMD virus (FMDV) lineages in the Southeast Asia region, and in particular identify possible sources of FMDV causing outbreaks in Malaysia. Complete VP1 sequences, obtained from 214 samples collected between 2000 and 2009, from FMD outbreaks in six Southeast Asian countries, were compared with sequences previously reported. Phylogenetic analysis of these sequences showed that there were two patterns of FMDV distribution in Malaysia. Firstly, for some lineages (O/SEA/Mya-98 and serotype A), outbreaks occurred every year in the country and did not appear to persist, suggesting that these incursions were quickly eradicated. Furthermore, for these lineages FMD viruses in Malaysia were closely related to those from neighbouring countries, demonstrating the close epidemiological links between countries in the region. In contrast, for O/ME-SA/PanAsia lineage, viruses were introduced and remained to cause outbreaks in subsequent years. In particular, the recent incursion and maintenance of the PanAsia-2 sublineage into Malaysia appears to be unique and independent from other outbreaks in the region. This study is the first characterisation of FMDV in Malaysia and provides evidence for different epidemiological sources of virus introduction into the country.

Keywords

Foot-and-mouth disease; VP1 sequencing; phylogenetics; Southeast Asia; Malaysia

2.1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious trans-boundary disease that affects the productivity of domesticated livestock in many countries of the world (Grubman and Baxt, 2004; Yang et al., 1999). FMD is caused by FMD virus (FMDV), a member of the genus *Aphthovirus* within the family *Picornaviridae*. FMDV is a small single-stranded, positive-sense RNA virus that is antigenically variable and can be divided into seven distinct serotypes; O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2 and SAT 3 (Brown, 2003). Serotypes O and A are widely distributed in many parts of Africa, Asia and South America. In contrast, the distribution of the remaining serotypes is not uniform across the FMD-endemic regions of the world: serotype Asia 1 is restricted to outbreaks in southern or central Asia, whereas SAT 1, SAT 2 and SAT 3 are normally geographically restricted to sub-Saharan Africa. FMD outbreaks due to serotype C have not been reported since 2004 (Roeder and Knowles, 2008). Phylogenetic analysis of molecular sequences of one of the outer capsid-coding genes, VP1 or 1D, can be used to serotype FMDVs and can also classify viruses further into topotypes reflecting their geographical distribution (Knowles and Samuel, 2003; Samuel and Knowles, 2001).

The endemic situation of FMD in Southeast Asia has been described previously, indicating that serotypes O, A and Asia1 are prevalent in the region (Gleeson, 2002; Rweyemamu et al., 2008). In addition, type C was reported in the Philippines between 1976 and 1994. Six countries in the region are endemic with the disease, Cambodia, Lao People's Democratic Republic (PDR), Peninsular Malaysia, Thailand, Myanmar and Vietnam, while Brunei, East Malaysia, Indonesia, Indonesia and Singapore are recognised internationally as FMD-free without vaccination

(Gleeson et al., 2003). Historically, FMD was also present in the Philippines, but since 1996 a vaccination programme has reduced reports of clinical disease (none have occurred since 2005). The country is following a pathway to establish FMD-free status: the Islands of Mindanao, Visayas, Palawan and Masbate, and two zones located on the Island of Luzon are FMD-free without vaccination, while the remaining zone on Luzon Island is currently FMD-free with vaccination.

Animal movements across international borders have been known to be an important factor in spreading the disease in the region (Ozawa, 1993). Establishing the genetic relationships between viruses collected from countries within the Southeast Asia region will help us to understand the pattern of FMD outbreaks that occur and links to animal movement may be revealed. In addition to characterising the broad FMD serotypes that are present in the region, it can also be important to identify specific genetic lineages that are responsible for the outbreaks in order to appreciate the complexity of the epidemiology of the disease. Therefore, the aim of this study was to use VP1 sequence data to characterise FMDV lineages in mainland Southeast Asia countries. These data have been used to reconstruct the relationship between viruses collected in Malaysia and neighbouring countries to determine whether FMDV enters Malaysia at regular intervals or alternatively if infection is maintained within the animal population in the country.

2.2. Material and method

2.2.1. FMD viruses

Viruses included in this study were selected from Southeast Asia countries (**Figure 2.1**) namely Malaysia (Peninsular Malaysia), Thailand, Myanmar, Cambodia, Laos and Vietnam collected from outbreaks between 2000 and 2009.

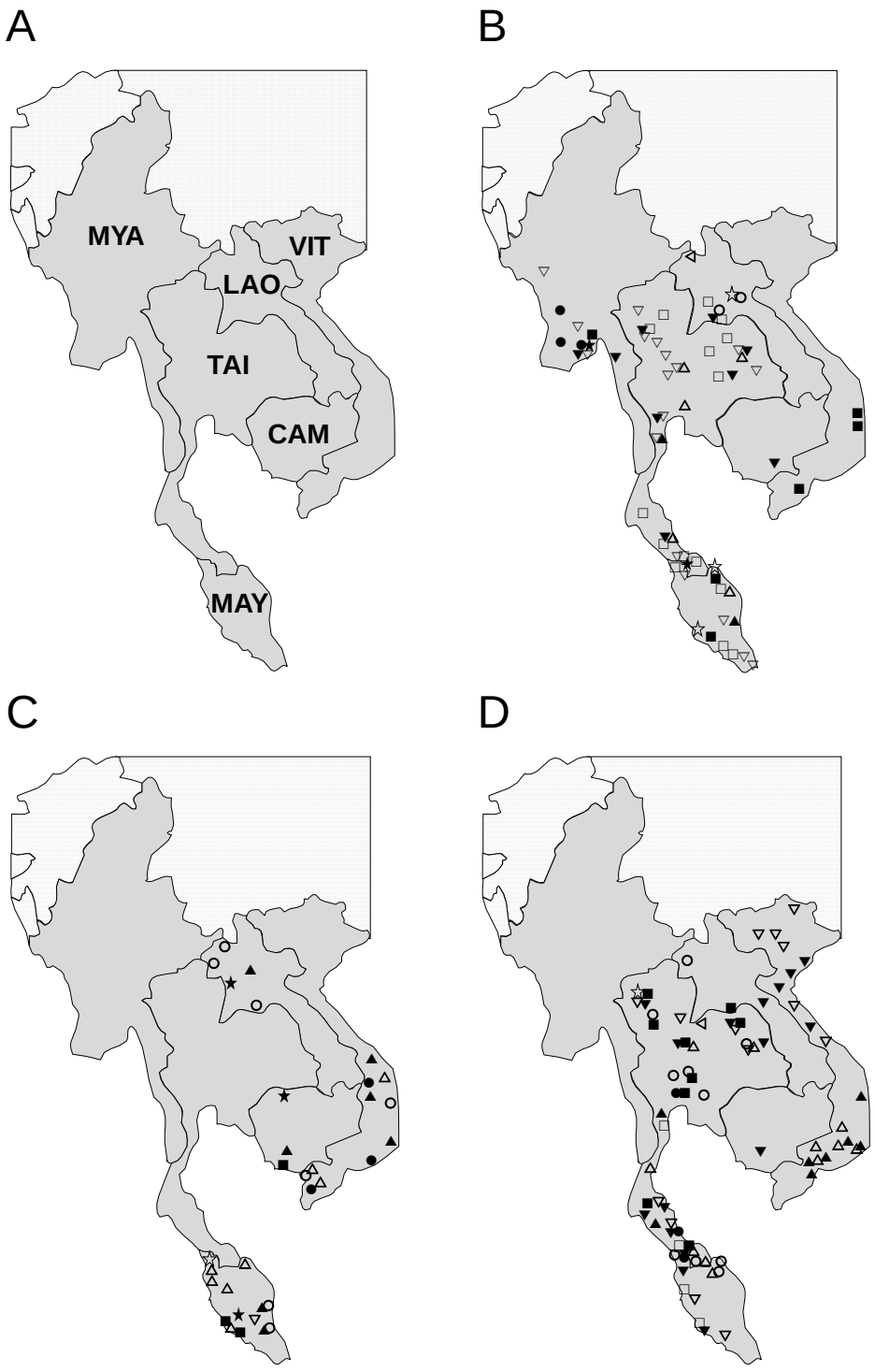


Figure 2.1: Maps showing [A] FMD endemic countries in Southeast Asia region (with 3 letter WRLFMD country codes); Myanmar (MYA), Thailand (TAI), Lao PDR (LAO), Vietnam (VIT), Cambodia (CAM) and Malaysia and distribution of [B] O/SEA/Mya-98, [C] O/ME-SA/PanAsia and [C] A/ASIA FMD outbreak of these countries from which FMD virus samples were analysed in this study. Symbols denote year of sample collection: 2000 (★), 2001 (☆), 2002 (●); 2003 (○); 2004 (▲); 2005 (△); 2006 (■); 2007 (□); 2008 (▼) and 2009 (▽). The dotted areas are neighbouring countries which are also endemic with FMD.

At the time of receipt to the World Reference Laboratory for FMD (WRLFMD, Institute for Animal Health), viruses from clinical samples was isolated in primary bovine thyroid cultures (BTy) and renal swine cell line (RS-2). The serotype for each isolate was determined by antigen typing ELISA (Ferris and Dawson, 1988). Samples were also tested using real time RT-PCR as previously described (Shaw et al., 2007). FMDV positive samples were further analysed for RT-PCR and VP1 sequencing. A brief overview of the FMD viruses used in this study and reference sequences employed in VP1 genetic typing analysis is provided in **Supplementary Tables S2.1 and S2.2**

2.2.2 VP1 RT-PCR and sequencing

Total RNA was extracted from tissue culture grown antigen by using RNeasy® Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK). One-step RT-PCR was carried out as described previously (Knowles et al., 2009). In order to cover diverse sequences, two forward primers were used: for serotype O, O-1C244F and O-1C272F while for serotype A, forward primers used were A-1C562F and A-1C612F. The reverse primer for both serotypes was EUR-2B52R. Reaction conditions for the RT-PCR were as described previously (Knowles et al., 2009; Knowles et al., 2005). PCR products were cleaned up using Illustra™ GFX™ PCR and Gel Band Purification Kit as recommended by the manufacturer. Cycle sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, Warrington, UK). Initially samples were sequenced using reverse sequencing primer NK72 and forward primer selected for sequencing was subsequently chosen based on NK72 sequence data. Primer details for RT-PCR and sequencing are provided in Table 2.1. Sequencing reactions were analysed using a ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

Table 2.1: List of oligonucleotides used for RT-PCR and sequencing

Primer	Primer sequence	Sense	Use
O-1C244F	GCA GCA AAA CAC ATG TCA AAC ACC TT	+	RT-PCR
O-1C272F	TBG CRG GNC TYG CCC AGT ACT AC	+	RT-PCR
A-1C562F	TAC CAA ATT ACA CAC GGG AA	+	RT-PCR/sequencing
A-1C612F	TAG CGC CGG CAA AGA CTT TGA	+	RT-PCR/sequencing
EUR-2B52R	GAC ATG TCC TCC TGC ATC TGG TTG AT	-	RT-PCR
NK72	GAA GGG CCC AGG GTT GGA CTC	-	Sequencing
O-1C499F	TAC GCG TAC ACC GCG TC	+	Sequencing
O-1C583F	GAC GGY GAY GCI CTG GTC GT	+	Sequencing
O-1C605eF	TAG CTA GCG CCGGCA AGG ACT TCG AG	+	Sequencing
O-1C605jF	TGG CCA GCG CTG GCA AAG ACT TTG AG	+	Sequencing
O-1C605kF	TGG CCA GCG ATG GCA AAG ACT TTG AG	+	Sequencing
O-1D293F	TGG AYA ACA CCA CYA AYC CAA C	+	Sequencing
O-1D296F	ACA ACA CCA CCA ACC CAA C	+	Sequencing
O-1D628R	GTT GGG TTG GTG GTG TTG T	-	Sequencing
O-1D628aR	GTT GGA TTA GTG GTG TTA T	-	Sequencing
O-1D487fR	TGA TGG CAC CGT AGT TGA A	-	Sequencing

2.2.3 Phylogenetic analysis

VP1 sequence data were assembled and analysed using SeqMan II (Lasergene 8.0; DNAStar Inc., WI, USA). This led to a final sequence of 639 nucleotides of the VP1 coding region. Sequence alignments were prepared using data for each FMDV serotype and included 87 reference sequences obtained from GenBank (19 sequences for serotype A and 68 sequences for serotype O) using the

ClustalW subroutine in BioEdit sequence alignment editor v.7.0 (Hall, 1999). Midpoint-rooted neighbor-joining (NJ) trees were constructed for each serotype using the Kimura 2-parameter nucleotide substitution model as implemented by MEGA 4.0 (Tamura et al., 2007). The robustness of the tree topology was assessed with 1000 bootstrap replicates as implemented within the program.

The sequence datasets were subjected to jModelTest 0.1.1 (Posada, 2008) to determine the most suitable nucleotide substitution model. Bayesian analysis was performed using the the BEAST software package v1.5.4 (Drummond and Rambaut, 2007). For each data-set, the maximum clade credibility (MCC) phylogenetic tree was inferred using the Bayesian Markov Chain Monte Carlo (MCMC) method. By incorporating the date of sample collection, the age of each virus was estimated. This technique has been proven to be useful in fine scale molecular forensic investigation to date back the origin of certain organisms or disease outbreak as showed in earlier studies (Oliveira et al., 2006; Siebenga et al., 2010; Zyl et al., 2010).

In BEAUti v1.5.4, the analysis utilised the general time reversible (GTR) model for substitution model with combination of gamma distribution and proportion of invariant sites (GTR+I+G) to describe rate heterogeneity among sites. In order to accommodate variation in substitution rate among branches, uncorrelated lognormal relaxed clock model was chosen for this analysis (Drummond et al., 2006). MCMC chains were 10^7 long and were performed in duplicate. BEAST output viewed with TRACER 1.5 (Drummond and Rambaut, 2007). Evolutionary trees were generated in the FigTree program v1.3.1 and trees from multiple run were combined using the LogCombiner v1.5.4 program.

2.3 Results

In total there were 121 serotype O and 93 serotype A viruses analysed in this study. NJ and Bayesian approaches revealed similar phylogenetic tree topologies: **Figures 2.2-2.7** show results from Bayesian analyses and **Supplementary Figures S2.1-S2.5** show the corresponding NJ trees. **Table 2.2** summarises the samples analysed in this study and the lineages concerned together with previous published sequences from outbreaks in Southeast Asian countries.

Table 2.2: Summary of sequences analysed in this study from FMD endemic countries in Southeast Asia (including GenBank sequences).

			Countries					
Year	Serotype	Topotype	Malaysia	Thailand	Laos	Cambodia	Myanmar	Vietnam
	O	SEA	-	-	(2)*	4(3)*	(2)*	-
1998	O	ME-SA	-	-	-	-	-	-
	A	ASIA	-	1	-	-	-	-
	O	SEA	-	(2)*	(1)*	-	(1)*	-
1999	O	ME-SA	-	(1)*	(2)*	-	-	-
	A	ASIA	-	2	-	-	-	-
	O	SEA	-	(1)*	-	-	(1)*	-
2000	O	ME-SA	2(1)*	-	(1)*	(2)*	-	-
	A	ASIA	-	-	-	-	-	-
	O	SEA	6(3)*	-	(2)*	-	-	-
2001	O	ME-SA	1	-	-	-	-	-
	A	ASIA	-	2	-	-	-	-
	O	SEA	4(1)*	-	-	-	5(1)*	-
2002	O	ME-SA	-	-	-	-	-	1(9)*
	A	ASIA	(1)*	2	-	-	-	-
	O	SEA	1	(1)*	(3)*	-	-	-
2003	O	ME-SA	2(1)*	(1)*	(4)*	-	-	2
	A	ASIA	4	6	(1)*	-	-	-
	O	SEA	-	1	-	-	1	-
2004	O	ME-SA	3	-	-	1	-	3(1)*
	A	ASIA	-	2	-	-	-	7
	O	SEA	2	4	-	-	-	2
2005	O	ME-SA	5	-	-	-	-	3
	A	ASIA	1	5	-	-	-	5
	O	SEA	1	-	-	-	1	4
2006	O	ME-SA	3	-	(4)*	2	-	-
	A	ASIA	-	10	(4)*	2	-	-
	O	SEA	5	10	4	-	-	-
2007	O	ME-SA	-	-	-	-	-	-
	A	ASIA	2	2	-	-	-	-
	O	SEA	-	6	3	-	2	-
2008	O	ME-SA	-	-	-	1	-	-
	A	ASIA	2	12	1	1		7
	O	SEA	6	17	1	-	5	-
2009	O	ME-SA	1	-	-	-	-	-
	A	ASIA	2	7	-	-	-	14(6)*

*Figures in brackets denote sequences retrieved from GenBank

2.3.1 Phylogenetic analysis of serotype O viruses

Analysis of the complete VP1 sequence data from recent FMDV samples together with the archived data from Southeast Asia countries revealed that they were clustered into three topotypes, as described previously, namely SEA (Southeast Asia), ME-SA (Middle East-South Asia) and CATHAY topotype (Gleeson et al., 2003; Knowles and Samuel, 2003). SEA was the most frequently detected type O topotype and had two lineages, named Mya-98 and Cam-94 (Gleeson et al., 2003). The MCC tree suggests that the Mya-98 and Cam-94 arose from a common ancestor approximately 35 years ago (**Figure 2.2**).

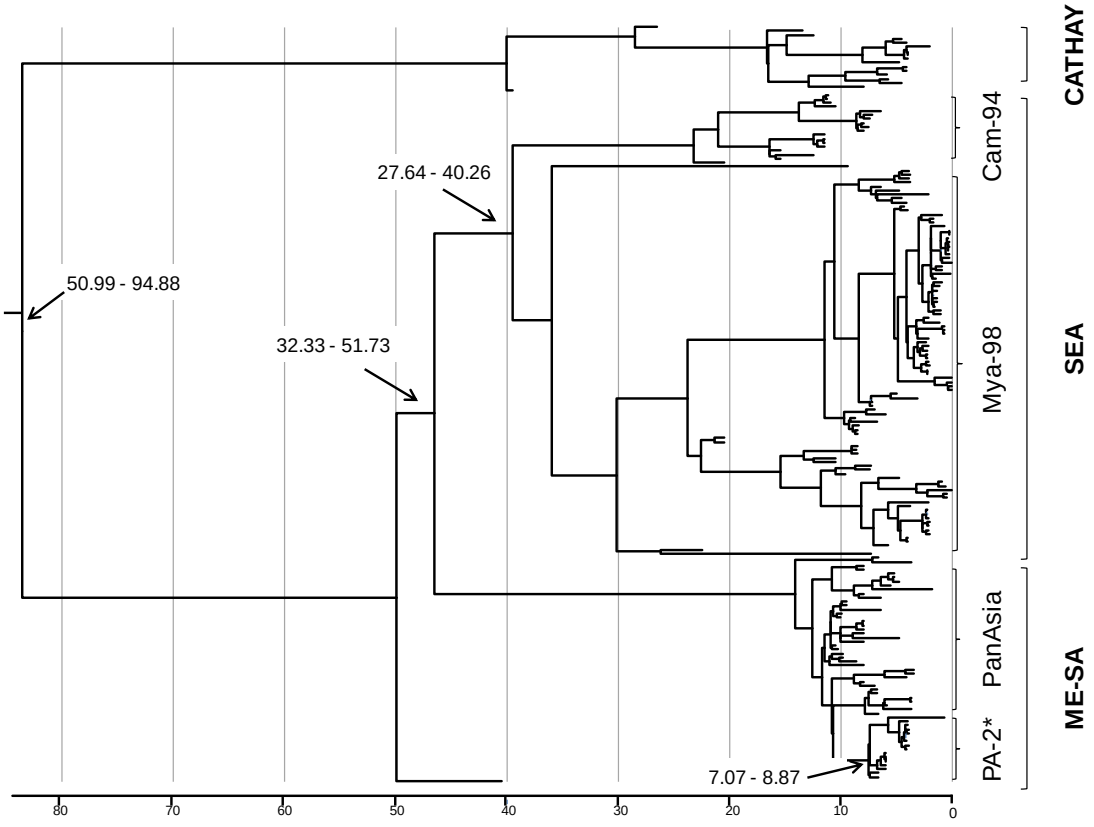


Figure 2.2: Maximum clade credibility (MCC) phylogenetic tree of serotype O with its lineages detected in Southeast Asia countries. The bold letters indicate the topotype (* = PanAsia-2). The X-axis is scale for years with vertical line indicating the age grid. The node labels show the estimated age range with 95% HPD (highest posterior density) of the most recent common ancestors for selected lineages.

Viruses belonging to the Mya-98 strain were detected in all six mainland countries of the region. Phylogenetic analysis showed that Malaysian isolates belonging to the O/SEA/Mya-98 lineage were interleaved with those from neighbouring countries in close groups which appeared to cluster based on the year of sampling (**Figure 2.3**). In some years, for example in 2001 (O/MAY/2/2001, O/MAY/3/2001 and O/MAY/4/2001) and 2009 (O/MAY/20/2009 and O/MAY/21/2009), clustering with viruses from neighbouring countries were supported by high posterior probability values (≥0.7) highlighted in the MCC tree as well as high bootstrap value in NJ trees. However, some genetic relationships were poorly supported, for example low posterior probability values can be seen in all samples from outbreaks in 2007. It was also apparent that when sequences from consecutive years were compared, virus isolates from outbreaks in Malaysia were not closely related to each other, rather Malaysian sequences largely grouped with contemporaneous sequences from neighbouring countries.

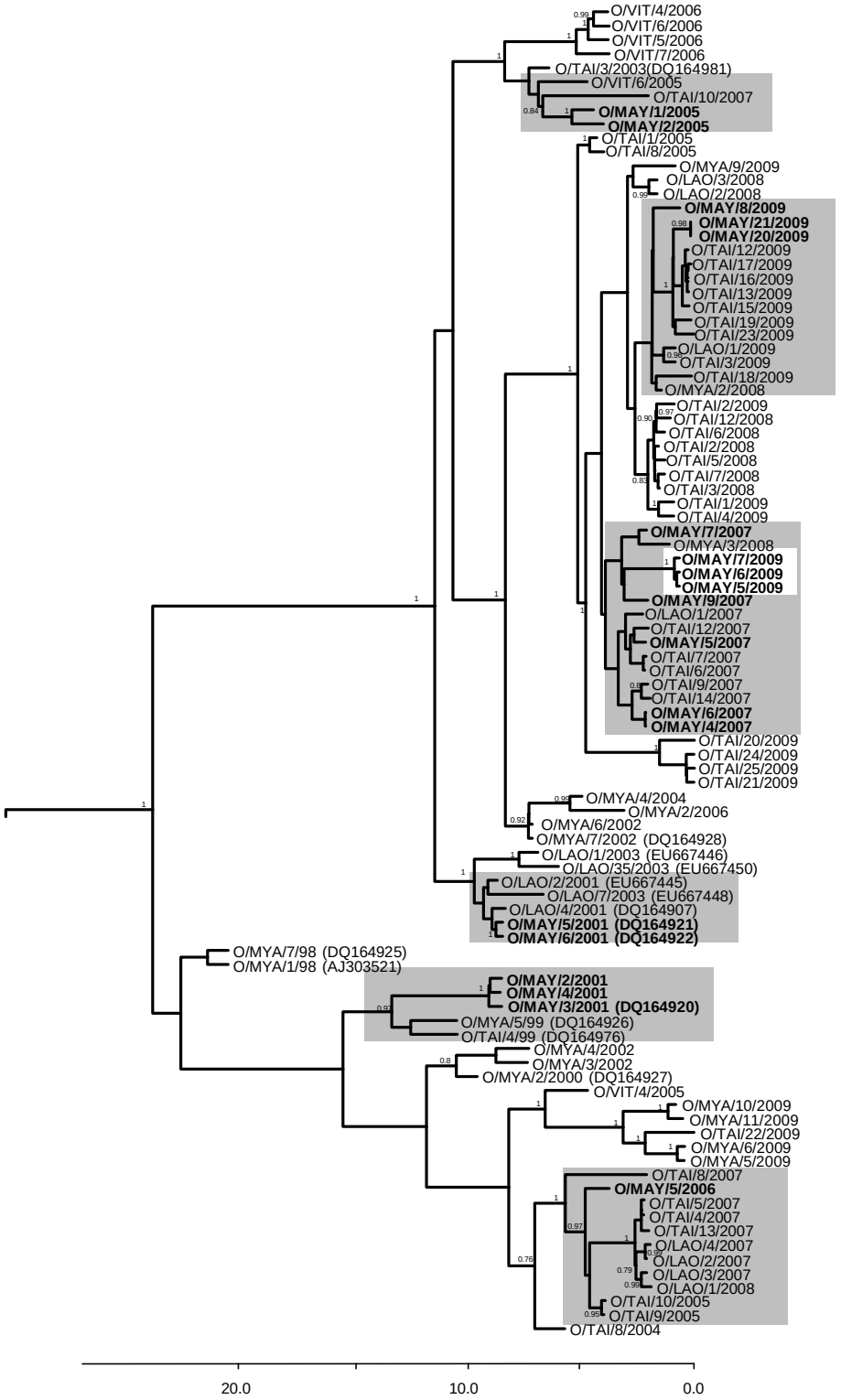


Figure 2.3: Maximum clade credibility (MCC) phylogenetic tree showing the Mya-98 strain of SEA topotype, serotype O. Malaysian samples are those in bold. Posterior probability values of more than 0.7 are indicated at the branch node. The X-axis is scale for years.

In contrast to the Mya-98 strain, the Cam-94 strain appeared only for a short period (between 1989 and 2003). Interestingly, this strain which was previously reported to be limited to Indo-China region has been detected in FMD outbreaks in Malaysia in three continuous years, from 2001 and 2003. These Malaysian isolates were clustered in one group and genetically closely related to viruses from outbreaks in Thailand and Lao PDR (**Figure 2.4**).

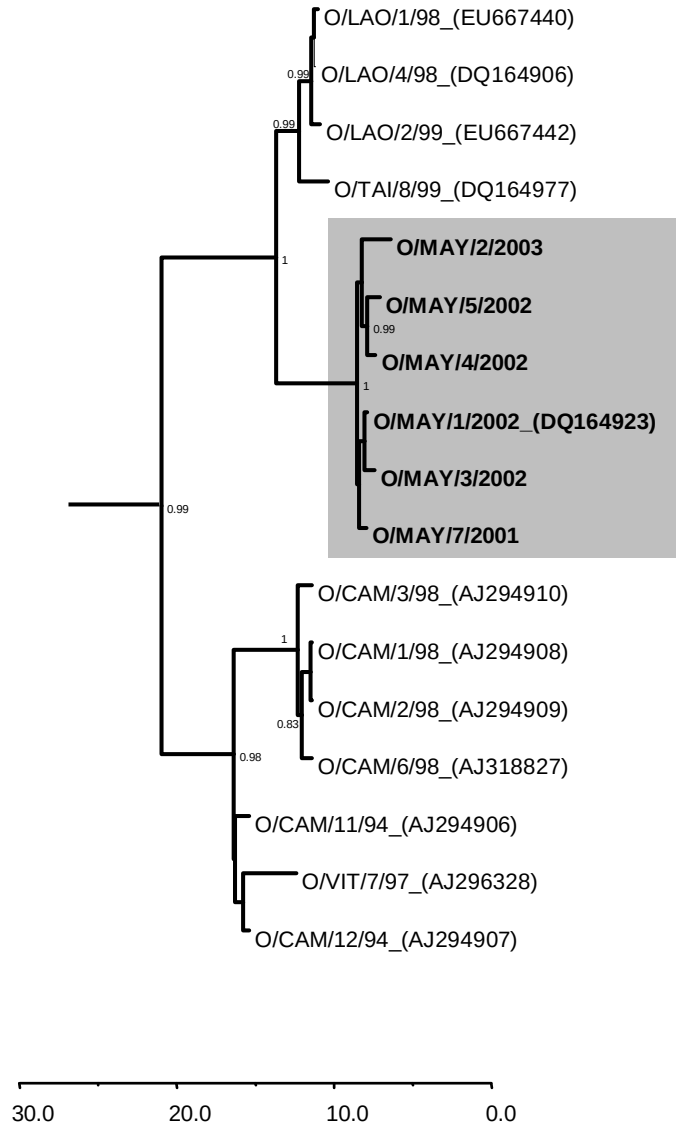


Figure 2.4: Maximum clade credibility (MCC) phylogenetic tree showing the Cam-94 strain of SEA topotype, serotype O. Malaysian samples are those in bold. Posterior probability values of more than 0.7 are indicated at the branch node. The X-axis is scale for years.

Another two FMDV lineages which have circulated in the region were PanAsia and PanAsia-2 within the ME-SA topotype (**Figure 2.5**). This topotype appeared to share a common ancestral history with the SEA topotype which dates back approximately between 32 to 51 years ago (**Figure 2.2**). In this study, viruses in the PanAsia group were detected in Malaysia in the year 2000 as well as in other countries in the region (Knowles et al., 2005). Three early examples of this topotype (years 2000 and 2001) from Malaysia were closely related to Vietnamese isolates and were broadly grouped with other isolates collected from the region. More recently FMDV from Malaysian outbreaks in 2003 to 2009 formed a well supported monophyletic cluster which significantly clustered with PanAsia-2 isolates detected in Southern Asia (Bhutan and Nepal). From the sequences analysed in this study, the new lineage, PanAsia-2 was appeared to emerge from PanAsia lineage approximately eight years ago.

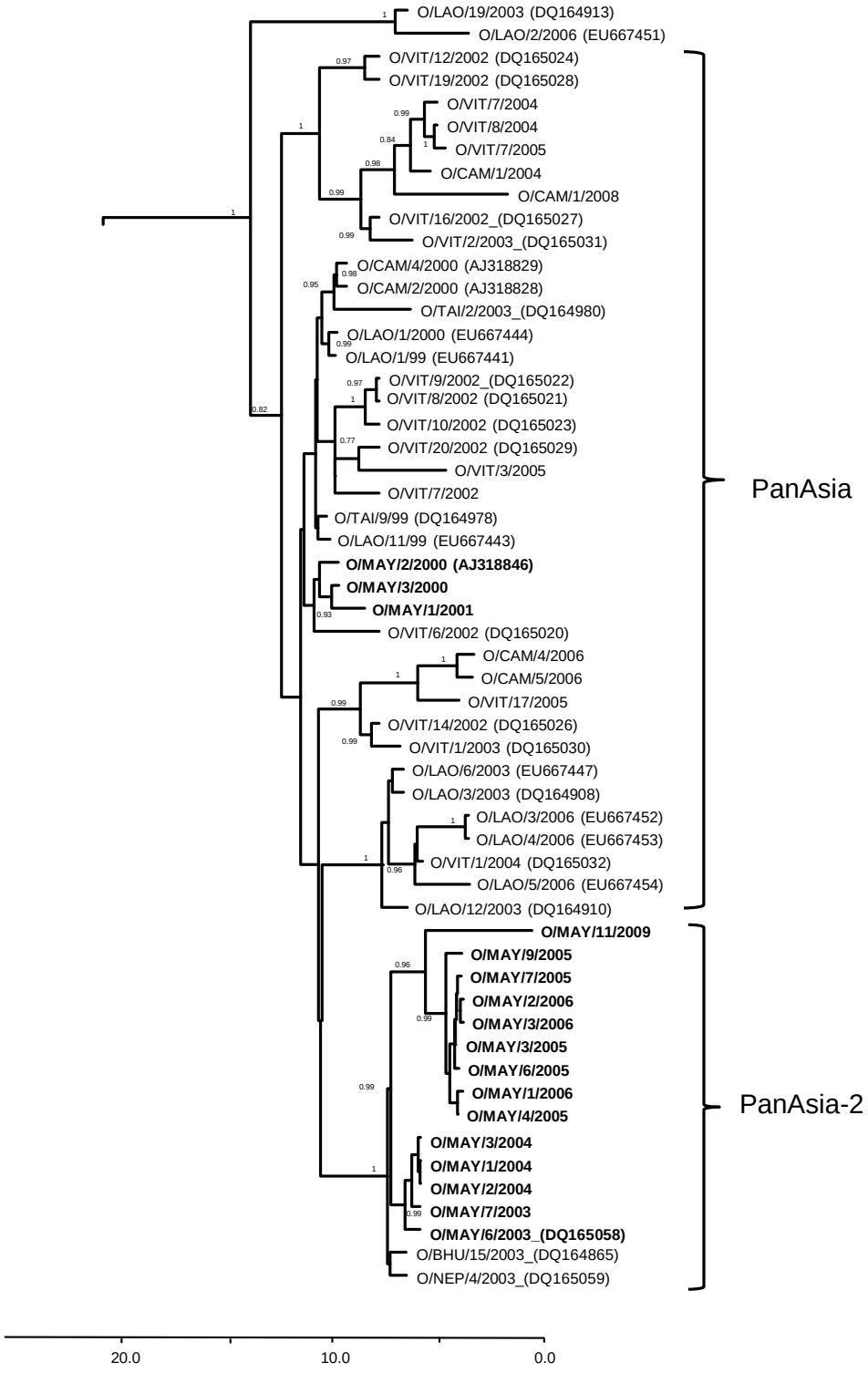


Figure 2.5: Maximum clade credibility (MCC) phylogenetic tree showing the PanAsia strain of ME-SA topotype, serotype O. Malaysian samples are those in bold. Posterior probability values of more than 0.7 are indicated at the branch node. The X-axis is scale for years.

CATHAY topotype viruses have been previously shown to be limited to Vietnam and Luzon Island of Philippines (Gleeson, 2002). In Malaysia, there was only a single outbreak in 2005 caused by this porcinophilic FMDV. In the same year, outbreaks due to this type were also reported in Thailand with viruses from both countries being closely related to those from Vietnam but unrelated to each other.

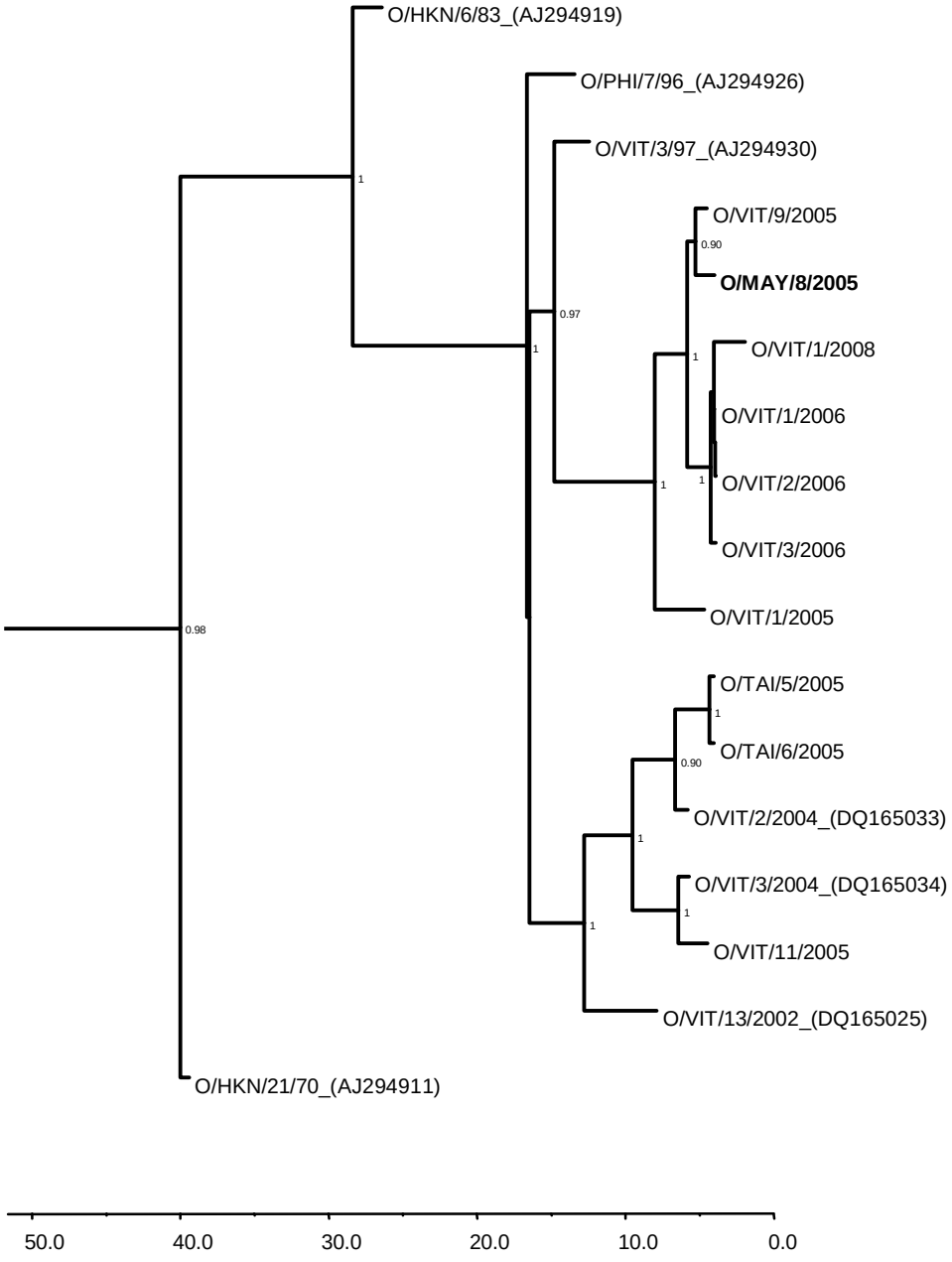


Figure 2.6: Maximum clade credibility (MCC) phylogenetic tree showing the CATHAY topotype, serotype O. Malaysian samples are those in bold. Posterior probability values of more than 0.7 are indicated at the branch node. The X-axis is scale for years.

2.3.2 Phylogenetic analysis of serotype A viruses

All of the viruses in this serotype were grouped in the ASIA topotype, the only topotype of serotype A viruses previously identified in the region (Knowles and Samuel, 2003). Viruses belonging to this serotype were occasionally reported in Malaysia (**Table 2.1**) and analysis showed a relationship with viruses collected from neighbouring countries (**Figure 2.7**). For example, samples from outbreaks in 2002 (A/MAY/2/2002) and 2009 (A/MAY/9/2009) were grouped with isolates collected in the same year from Thailand and Vietnam, respectively; clustering that was supported with posterior probability values of 0.9 to 1.0. It is interesting to note that sequences from outbreaks in different years in Malaysia fell into new distinct clusters which were distributed throughout the phylogenetic tree.

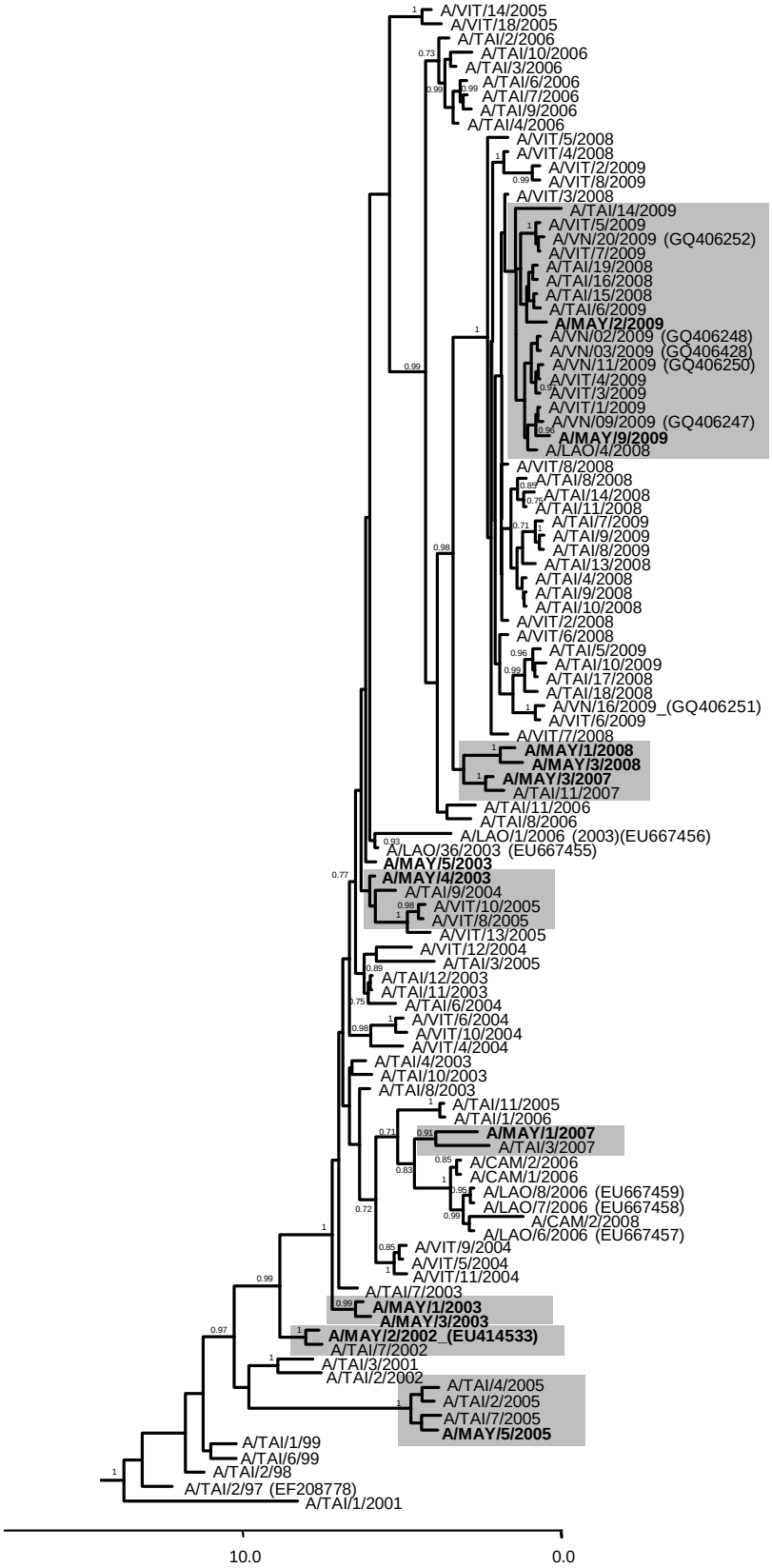


Figure 2.7: Maximum clade credibility (MCC) phylogenetic tree showing the serotype A. Malaysian samples are those in bold. Posterior probability values of more than 0.7 are indicated at the branch node. The X-axis is scale for years.

2.4 Discussion

Phylogenetic analysis of VP1 sequences are widely used to identify and characterise FMDV lineages (Klein et al., 2006; Knowles et al., 2009; Konig et al., 2007); however, description of the molecular epidemiology of FMDV in Southeast Asia (SEA) has not been as extensive as some other regions in the world. In order to define the relationship between viruses circulating in SEA, this report describes the results of phylogenetic analyses using Neighbor-joining (Tamura et al., 2007) and Bayesian methods (Drummond and Rambaut, 2007) of VP1 sequence data from FMDV samples collected in different countries in the region. A particular focus of this study was to determine the genetic relationship between FMDV collected from Malaysia and compare these with those sampled from other countries in SEA region. These data can be used to determine whether FMDVs are introduced year-on-year into the country or alternatively are maintained in Malaysia as distinct lineages to causes outbreaks over many years. However, by themselves these trees cannot define specific routes of transmission between countries and it is important to highlight that Malaysian samples analysed in this study were only small proportion of the actual FMD outbreaks reported in the country as shown in **Figure 2.8**. This situation (lack of sampling and laboratory analysis) has been identified as one of the constraints to control of FMD in the region and strategies to overcome this problem have been proposed in the SEAFMD 2020 roadmap (Murray, 2006) (details available at http://www.seafmd-rcu.oie.int/index.php).

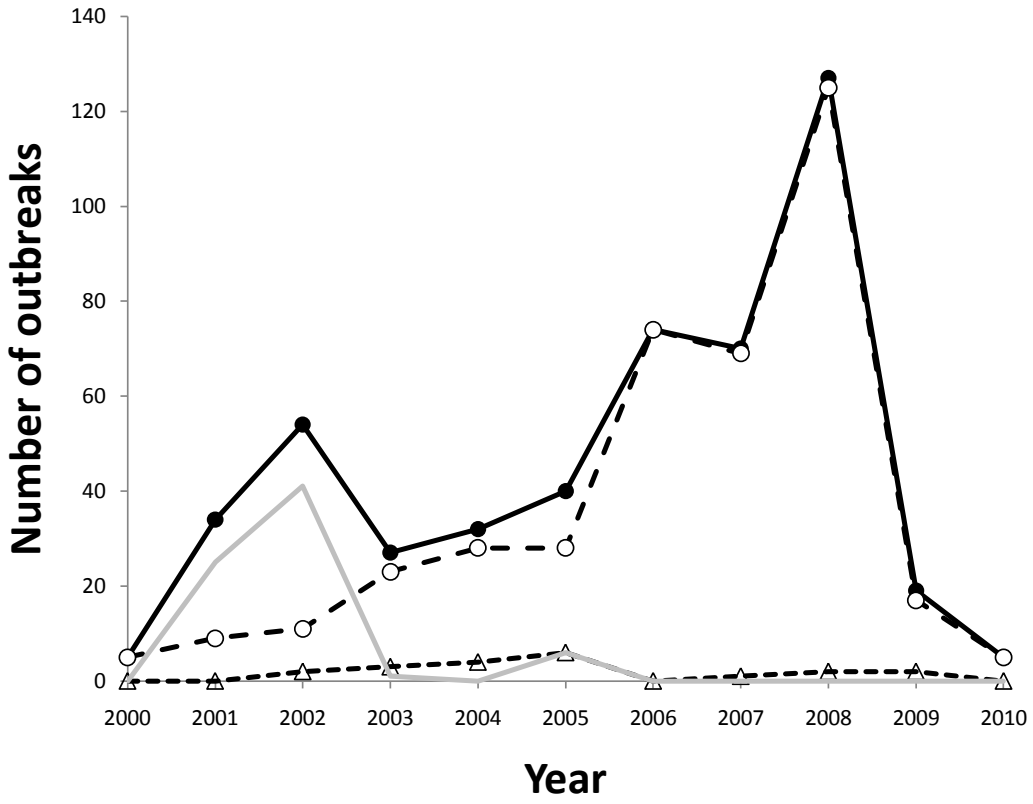


Figure 2.8: Temporal distribution of FMD outbreaks in Malaysia (2000-2010) from http://www.arahis.oie.int/reports.php. Points represent total FMD outbreaks reported in the country (●) and those typed as serotype O (○) and serotype A (△) (serotyping was carried out in the National Foot-and-Mouth Disease Laboratory Malaysia). Untyped outbreaks in Malaysia are shown by a grey line. Note: data for 2010 only includes reports up until June.

Previously, seven distinct FMDV lineages have been recognised in Southeast Asia: serotype O/SEA topotype (Mya-98 and Cam-94 strains); O/ME-SA topotype (PanAsia and the derivative Pan-Asia-2 strain) and O/CATHAY topotype, serotype A (ASIA topotype) and serotype Asia 1. In this study, the O/SEA/Mya-98 lineage of the SEA topotype and serotype A (ASIA topotype) viruses were the most frequent type of FMDV characterised supporting work from previous studies (Gleeson et al., 2003; Khounsy et al., 2009; Le et al., 2009; Le et al., 2010b; Rweyemamu et al., 2008). Interestingly, phylogenetic analysis revealed two different and distinctive

patterns for the molecular epidemiology of FMD in Malaysia. Firstly for Mya-98 (serotype O) and serotype A, viruses were interleaved with sequences of other samples collected from the region. In contrast, there was evidence that viruses come from ME-SA topotype (serotype O) were maintained within Malaysia and formed discrete lineages that were separate from other viruses in Southeast Asian countries. This second pattern was particularly evident for PanAsia-2 strain which was only recognised in Malaysia, and where isolates from 2003-2009 formed a monophyletic group.

The importance of the movement of infected animals as a mechanism responsible for the spread of FMD among countries in SEA region has been highlighted previously (Gleeson, 2002; Gleeson et al., 2003; Ozawa, 1993). These movements occur within countries or across borders from Laos, Myanmar and Cambodia towards Thailand and Malaysia driven by a higher demand and market price. Apart from cattle, Malaysia has also imported suckling pigs from Vietnam for many years which could pose another source of infection to the country (Isa, 2007). Malaysia being an animal and animal product importer as well as part of Malaysia-Thailand-Myanmar (MTM) peninsular control zone is vulnerable to introduction of infected animals (Wongsathapornchai et al., 2008). For serotype O (Mya-98 and CATHAY) as well as Serotype A the sequence data suggests that the most likely source of infection in Malaysia was via entrance of infected animals or animal products into the country. In fact two isolates (A/MAY/2/2002 and O/MAY/5/2007) presented in this study were actually reported in quarantined animals in a quarantine station located in the northern part of the country after being imported from a neighbouring country.

The global distribution of the pandemic PanAsia strain has been described previously in South Asia before it spread widely to other part such as the Middle East and Southeast Asia (Hemadri et al., 2002; Knowles et al., 2005). The earliest examples of the PanAsia strain in Malaysia (2000-2001) had sequences that were closely related to isolates from Vietnam, findings that are consistent with proposed transmission of this virus in the region via trade route of suckling pigs from Vietnam (Gleeson et al., 2003). More recently (2003-2009), PanAsia viruses have continued to cause outbreaks in Malaysia. This so called PanAsia-2 strain is widely spread in southern Asia (Indian sub-continent including Bhutan and Nepal) and the Middle East, but in Southeast Asia is unique to Malaysia and genetically discrete from earlier viruses in the country. There are no legal animal movements from the Indian sub-continent into Malaysia, however, at least 80% of the imports of beef and buffalo meat into the country for domestic consumption originates from India (Gleeson et al., 2003). Therefore, although formal links have not been established, the sequence data suggests that a single introduction of PanAsia-2 occurred probably from a country in South Asia and that this lineage has been maintained in the country for the past eight years.

These phylogenetic relationships can be used to indicate whether FMD viruses are introduced on a regular basis, or alternatively are maintained for long periods of time within Malaysia. Interestingly, the broad interpretation of the trees differs between lineages (particularly for O/SEA/Mya-98 and O/ME-SA/PanAsia viruses). One explanation for these findings is that different host-species interactions may impact upon the epidemiology of these discrete lineages in the region. However, in light of the lack of complete epidemiological information for FMD in Malaysia and limited number of viruses available in this study, further analysis are required

that includes sequence data from other potential source countries outside of SEA (such as southern Asia) over a similar time course before this conclusion can be considered reliable.

Further collection and analysis of samples, together with improved local epidemiological investigation of FMD outbreaks in countries in the Southeast Asia region is required to improve our understanding of the epidemiology of FMD in the region. O/SEA/Mya-98 and serotype A lineages have recently spread beyond the Southeast Asian region to cause outbreaks in East Asia countries including countries which were previously FMD-free (People's Republic of China and Republic of Korea for serotype A and in addition Hong Kong, Japan and Mongolia for Mya-98) (Paton et al., 2010). Although focussing on serotype O and A, serotype Asia-1 also contributes to disease outbreaks in the region. In Malaysia, only two outbreaks caused by serotype Asia 1 which were reported in 1997 and 1999 respectively, consistent with this serotype being only sporadically reported in the Southeast Asia region in the past ten years (Valarcher et al., 2009). Outbreaks due to this serotype have been reported recently in China and Vietnam (Valarcher et al., 2005; Valarcher et al., 2009) suggesting that this virus can also spread rapidly across Asia. Therefore, although this serotype was not detected in this study and no outbreaks due to this serotype have been reported in Malaysia for more than 10 years (data from National Foot-and-mouth Disease Laboratory, Kota Bharu, Malaysia), it is important to consider the potential threat posed by this serotype due to importation of live animals from affected countries such as P. R. China.

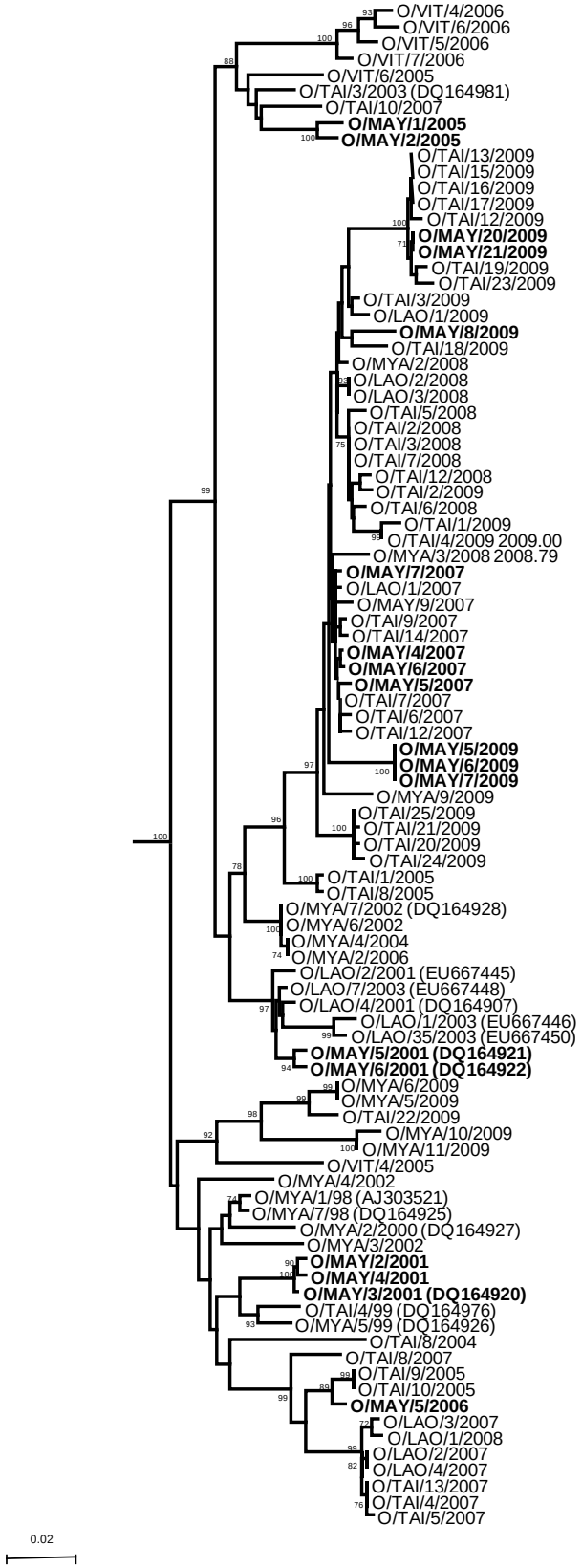
In conclusion, this study provides an overview of the molecular epidemiology of FMDV in Southeast Asia. Although only a limited number of ad-hoc samples were available for sequence analysis, this study highlights the spread of

FMDV between countries in Southeast Asia. Interpretation of data used in this study is limited by field epidemiological data and records of animal movements across international borders. The spread of FMDV between countries in Southeast Asia shown in this study demonstrates the need for active surveillance and strengthening of animal movement regulations across borders. In addition, a continuous good regional network is essential in order to ensure the sustainability of the existing control programmes.

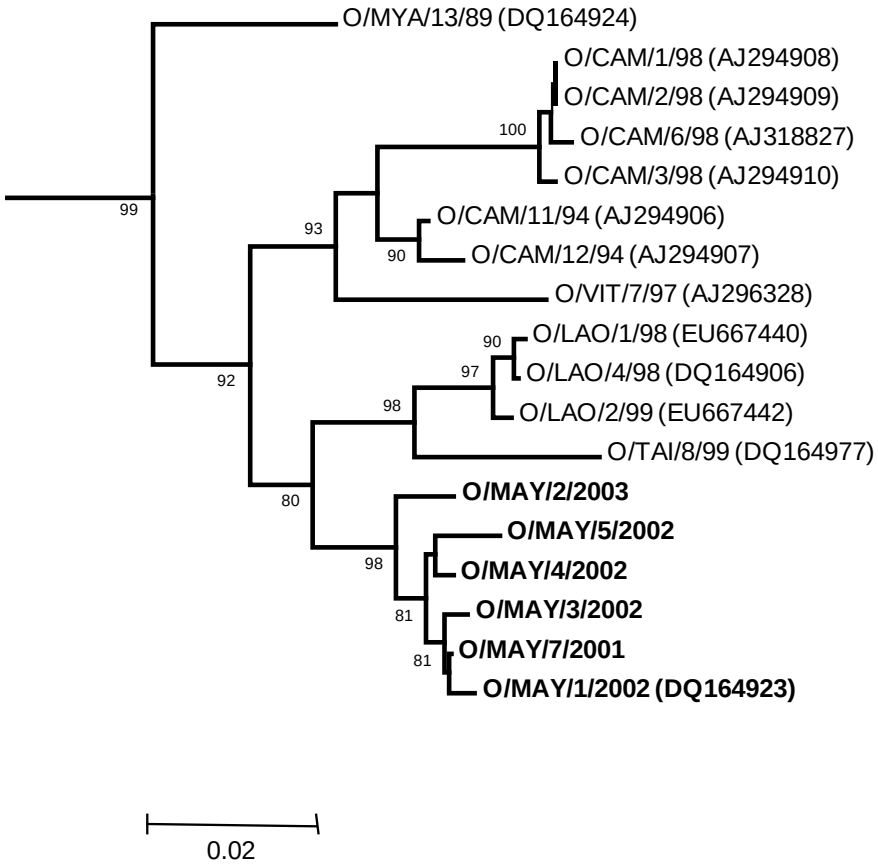
Acknowledgements

The authors thank all colleagues in the Department of Veterinary Services Malaysia and Dr Norlida Othman of National Foot-and-Mouth Disease Laboratory, Kelantan, Malaysia for providing samples and data from Malaysian outbreaks, as well as Regional Reference Laboratory (RRL) Pakchong, Thailand and all other Southeast Asia for the additional samples used in this study. In addition, gratitude is extended to Dr Oliver Pybus for providing helpful advice for the BEAST analysis. This study was carried out at Institute for Animal Health, Pirbright Laboratory, UK and was sponsored by Department of Public Services of the Malaysian Government and supported by the Department for Environment, Food and Rural Affairs, UK (project numbers SE2935 and SE2939).

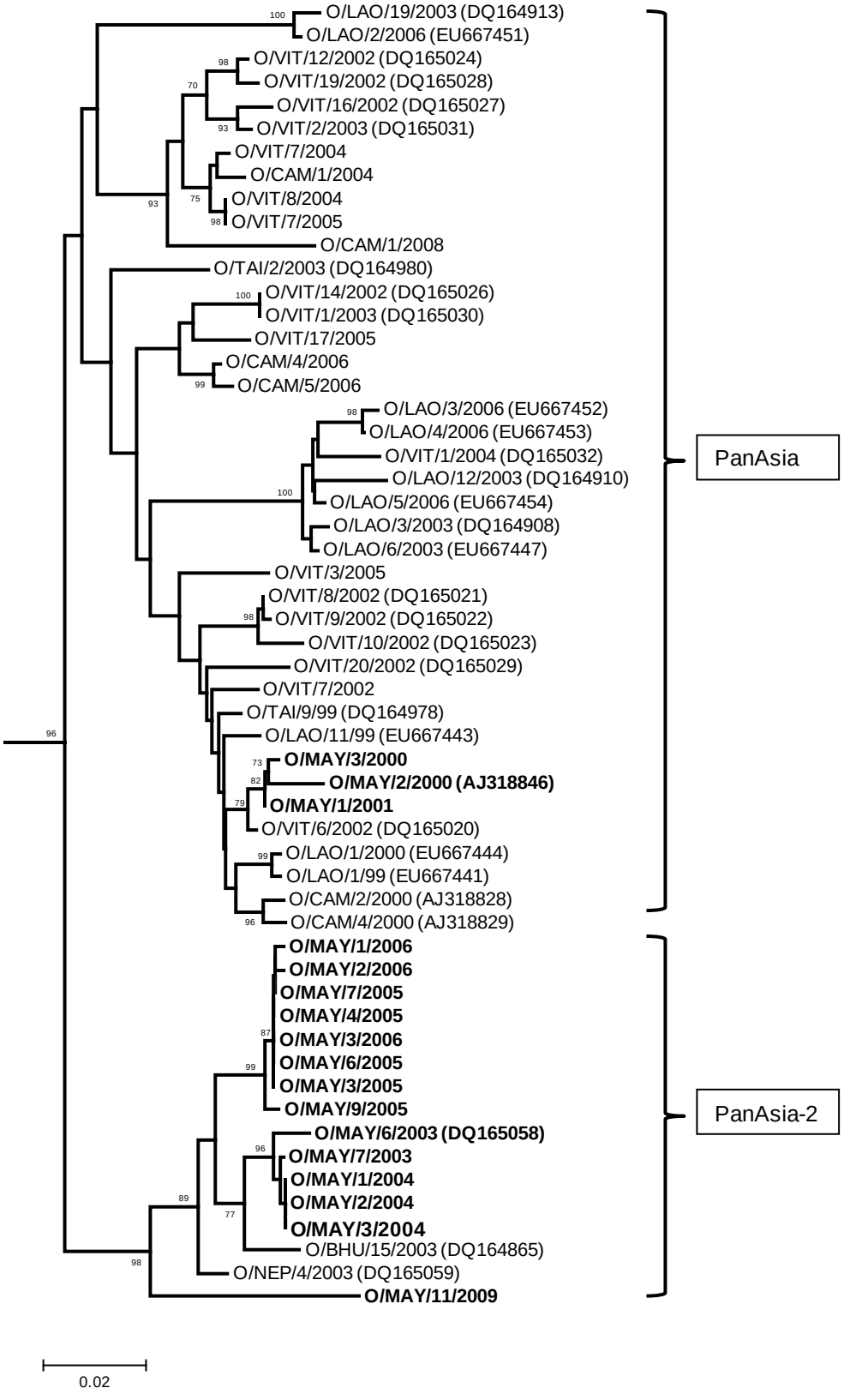
Supplementary Figures and Tables for Chapter 2



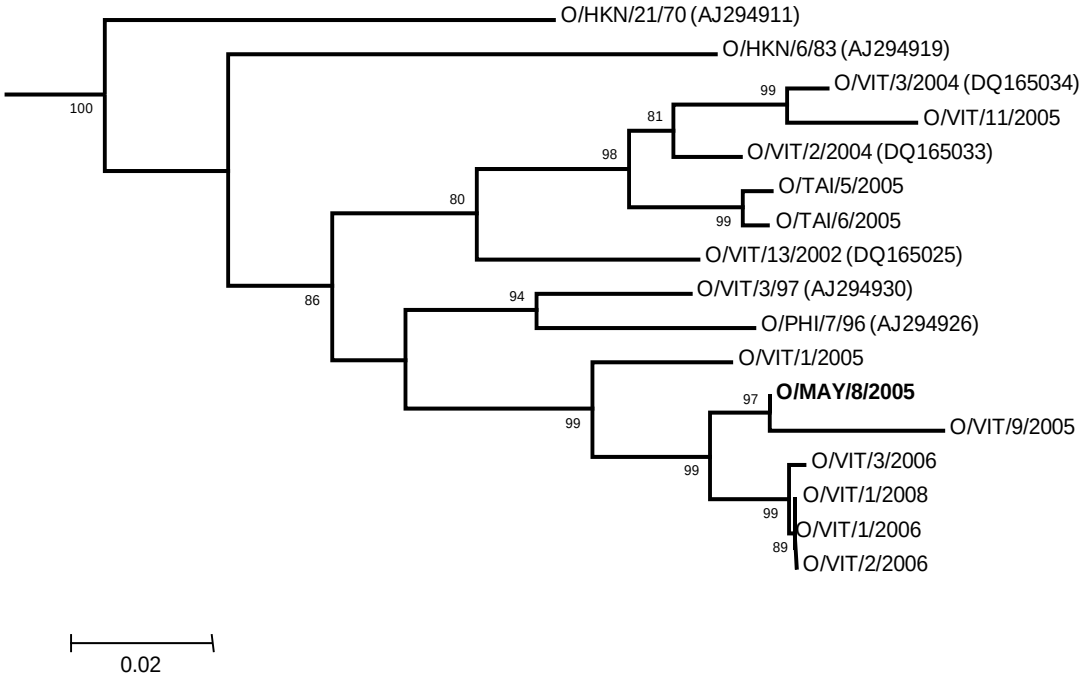
Supplementary Figure S2.1: Midpoint-rooted Neighbor-joining phylogenetic tree of Mya-98 strain of SEA topotype, serotype O. Only bootstrap values ≥ 70% are shown.



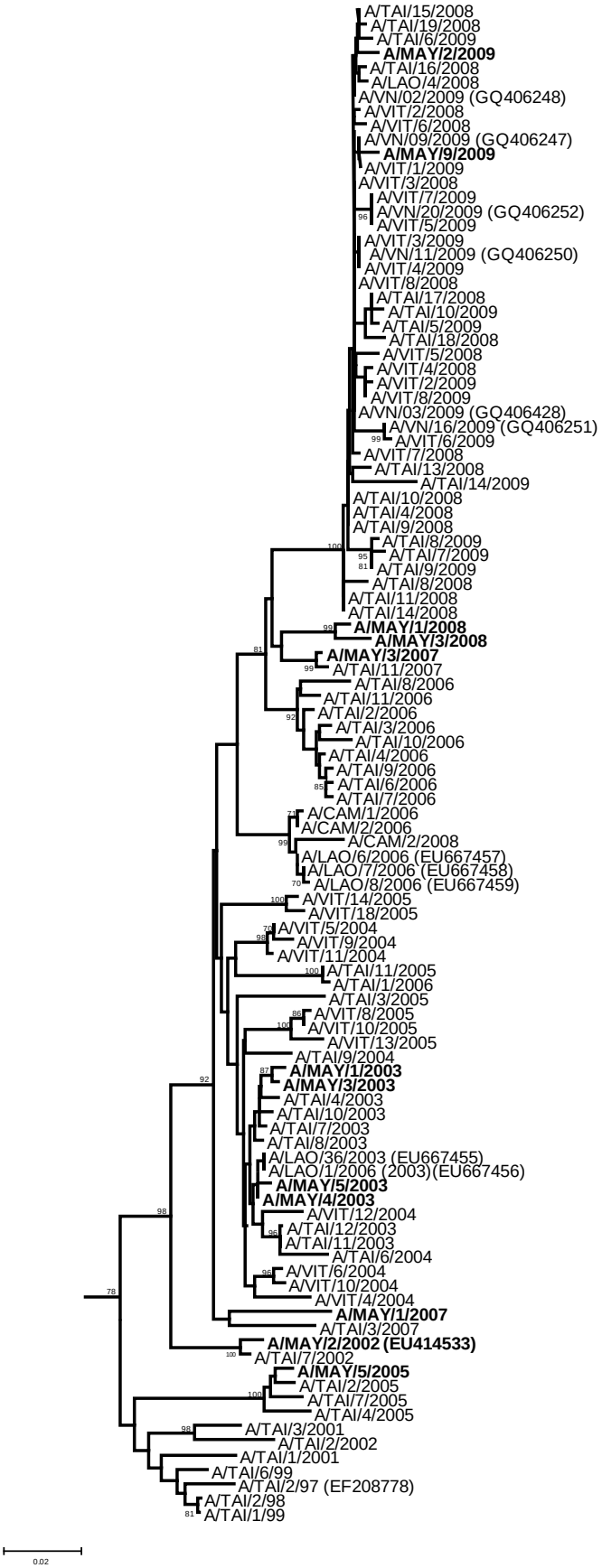
Supplementary Figure S2.2: Midpoint-rooted Neighbor-joining phylogenetic tree of Cam-94 strain of SEA topotype, serotype O. Only bootstrap values ≥ 70% are shown.



Supplementary Figure S2.3: Midpoint-rooted Neighbor-joining phylogenetic tree of PanAsia and PanAsia-2 strain of ME-SA topotype, serotype O. Only bootstrap values ≥ 70% are shown.



Supplementary Figure S2.4: Midpoint-rooted Neighbor-joining phylogenetic tree of CATHAY topotype, serotype O. Only bootstrap values ≥ 70% are shown.



Supplementary Figure S2.5: Midpoint-rooted Neighbor-joining phylogenetic tree of serotype A. Only bootstrap values ≥ 70% are shown.

Supplementary Table S2.1: FMD viruses from outbreak in Southeast Asia countries used in this study.

No	Sample ID	Country	District/Province	Collection date	Species	Serotype	Topotype	Strain	Accession no
1	O/CAM/1/2004	Cambodia	-	23/06/2004	Cattle	O	ME-SA	PanAsia	HQ116171
2	O/CAM/4/2006	Cambodia	-	28/07/2006	Cattle	O	ME-SA	PanAsia	HQ116172
3	O/CAM/5/2006	Cambodia	-	28/06/2006	Cattle	O	ME-SA	PanAsia	HQ116173
4	O/CAM/1/2008	Cambodia	Srang, Kandal	28/02/2008	Cattle	O	ME-SA	PanAsia	HQ116174
5	O/LAO/1/2007	Laos	Xayaboury	24/08/2007	Buffalo	O	SEA	Mya-98	HQ116175
6	O/LAO/2/2007	Laos	Vientiane	19/10/2007	Buffalo	O	SEA	Mya-98	HQ116176
7	O/LAO/3/2007	Laos	Vientiane	19/10/2007	Cattle	O	SEA	Mya-98	HQ116177
8	O/LAO/4/2007	Laos	Vientiane	13/12/2007	Cattle	O	SEA	Mya-98	HQ116178
9	O/LAO/1/2008	Laos	Vientiane	03/01/2008	Cattle	O	SEA	Mya-98	HQ116179
10	O/LAO/2/2008	Laos	Sikhottabong, Vientian Capital	02/04/2008	Pig	O	SEA	Mya-98	HQ116180
11	O/LAO/3/2008	Laos	Sikhottabong, Vientian Capital	02/04/2008	Pig	O	SEA	Mya-98	HQ116181
12	O/LAO/1/2009	Laos	Phonethung, Champasak	22/01/2009	Buffalo	O	SEA	Mya-98	HQ116182
13	O/MAY/3/2000	Malaysia	Gombak, Selangor	11/02/2000	Pig	O	ME-SA	PanAsia	HQ116183
14	O/MAY/1/2001	Malaysia	Pokok Sena, Kedah	13/05/2001	Cattle	O	ME-SA	PanAsia	HQ116184
15	O/MAY/2/2001	Malaysia	Kuala Langat, Selangor	22/06/2001	Cattle	O	SEA	Mya-98	HQ116185
16	O/MAY/4/2001	Malaysia	Kuala Langat, Selangor	01/06/2001	Cattle	O	SEA	Mya-98	HQ116186
17	O/MAY/7/2001	Malaysia	Kulim, Kedah	28/12/2001	Cattle	O	SEA	Cam-94	HQ116187
18	O/MAY/3/2002	Malaysia	Bukit Mertajam, Penang	02/07/2002	Cattle	O	SEA	Cam-94	HQ116188
19	O/MAY/4/2002	Malaysia	Bandar Baru, Kedah	22/07/2002	Cattle	O	SEA	Cam-94	HQ116189
20	O/MAY/5/2002	Malaysia	Besut, Terengganu	23/10/2002	Cattle	O	SEA	Cam-94	HQ116190
21	O/MAY/2/2003	Malaysia	Tasek Gelugor, Penang	02/07/2003	Cattle	O	SEA	Cam-94	HQ116191
22	O/MAY/7/2003	Malaysia	Kuantan, Pahang	29/12/2003	Cattle	O	ME-SA	PanAsia-2	HQ116192
23	O/MAY/1/2004	Malaysia	Rompin, Pahang	01/01/2004	Cattle	O	ME-SA	PanAsia-2	HQ116193
24	O/MAY/2/2004	Malaysia	Kuantan, Pahang	02/01/2004	Cattle	O	ME-SA	PanAsia-2	HQ116194
25	O/MAY/3/2004	Malaysia	Pekan, Pahang	08/01/2004	Cattle	O	ME-SA	PanAsia-2	HQ116195
26	O/MAY/1/2005	Malaysia	Kuala Terengganu, Terengganu	22/06/2005	Cattle	O	SEA	Mya-98	HQ116196
27	O/MAY/2/2005	Malaysia	Tanah Merah, Kelantan	02/12/2005	Cattle	O	SEA	Mya-98	HQ116197
28	O/MAY/3/2005	Malaysia	Kota Setar, Kedah	29/09/2005	Cattle	O	ME-SA	PanAsia-2	HQ116198
29	O/MAY/4/2005	Malaysia	Tapah, Perak	20/10/2005	Cattle	O	ME-SA	PanAsia-2	HQ116199
30	O/MAY/6/2005	Malaysia	Sungai Bakap, Penang	11/11/2005	Pig	O	ME-SA	PanAsia-2	HQ116200
31	O/MAY/7/2005	Malaysia	Sungai Bakap, Penang	15/12/2005	Cattle	O	ME-SA	PanAsia-2	HQ116201
32	O/MAY/8/2005	Malaysia	Kuala Langat, Selangor	02/12/2005	Pig	O	Cathay	unnamed	HQ116202
33	O/MAY/9/2005	Malaysia	Lenggong, Perak	30/12/2005	Cattle	O	ME-SA	PanAsia-2	HQ116203
34	O/MAY/1/2006	Malaysia	Sepang, Selangor	20/01/2006	Cattle	O	ME-SA	PanAsia-2	HQ116204
35	O/MAY/2/2006	Malaysia	Kuala Langat, Selangor	27/01/2006	Cattle	O	ME-SA	PanAsia-2	HQ116205

36	O/MAY/3/2006	Malaysia	Serdang, Selangor	27/01/2006	Cattle	O	ME-SA	PanAsia-2	HQ116206
37	O/MAY/5/2006	Malaysia	Pasir Puteh, Kelantan	27/02/2006	Cattle	O	SEA	Mya-98	HQ116207
38	O/MAY/4/2007	Malaysia	Kuala Pilah, Negeri Sembilan	30/09/2007	Cattle	O	SEA	Mya-98	HQ116208
39	O/MAY/5/2007	Malaysia	Padang Besar, Perlis	04/10/2007	Cattle	O	SEA	Mya-98	HQ116209
40	O/MAY/6/2007	Malaysia	Besut, Terengganu	10/10/2007	Cattle	O	SEA	Mya-98	HQ116210
41	O/MAY/7/2007	Malaysia	Jasin, Melaka	20/10/2007	Cattle	O	SEA	Mya-98	HQ116211
42	O/MAY/9/2007	Malaysia	Pasir Puteh, Kelantan	07/11/2007	Cattle	O	SEA	Mya-98	HQ116212
43	O/MAY/5/2009	Malaysia	Kluang, Johor	29/03/2009	Cattle	O	SEA	Mya-98	HQ116213
44	O/MAY/6/2009	Malaysia	Kluang, Johor	29/03/2009	Cattle	O	SEA	Mya-98	HQ116214
45	O/MAY/7/2009	Malaysia	Kluang, Johor	29/03/2009	Cattle	O	SEA	Mya-98	HQ116215
46	O/MAY/8/2009	Malaysia	Kota Tinggi, Johor	07/04/2009	Cattle	O	SEA	Mya-98	HQ116216
47	O/MAY/11/2009	Malaysia	Temerloh, Pahang	26/04/2009	Cattle	O	ME-SA	PanAsia-2	HQ116217
48	O/MAY/20/2009	Malaysia	Chuping, Perlis	25/09/2009	Cattle	O	SEA	Mya-98	HQ116218
49	O/MAY/21/2009	Malaysia	Chuping, Perlis	25/09/2009	Cattle	O	SEA	Mya-98	HQ116219
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53	O/MYA/6/2002	Myanmar	Yangon	23/10/2002	Pig	O	SEA	Mya-98	HQ116223
54	O/MYA/4/2004	Myanmar	-	27/12/2004	Cattle	O	SEA	Mya-98	HQ116224
55	O/MYA/2/2006	Myanmar	-	25/10/2006	Cattle	O	SEA	Mya-98	HQ116225
56	O/MYA/2/2008	Myanmar	-	16/06/2008	Cattle	O	SEA	Mya-98	HQ116226
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67	O/TAI/8/2005	Thailand	Petchaboon	05/12/2005	Cattle	O	SEA	Mya-98	HQ116237
68	O/TAI/9/2005	Thailand	Pattalung	05/12/2005	Cattle	O	SEA	Mya-98	HQ116238
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75	O/TAI/9/2007	Thailand	Lumphun	07/11/2007	Cattle	O	SEA	Mya-98	HQ116245
76	O/TAI/10/2007	Thailand	Lampang	14/11/2007	Cattle	O	SEA	Mya-98	HQ116246
77	O/TAI/12/2007	Thailand	Suratthani	20/11/2007	Cattle	O	SEA	Mya-98	HQ116247
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79	O/TAI/14/2007	Thailand	Udonthani	19/12/2007	Cattle	O	SEA	Mya-98	HQ116249
80	O/TAI/2/2008	Thailand	Ratchaburi	27/04/2008	Cattle	O	SEA	Mya-98	HQ116250
81	O/TAI/3/2008	Thailand	Ratchaburi	10/05/2008	Cattle	O	SEA	Mya-98	HQ116251
82	O/TAI/5/2008	Thailand	Pattalung	11/08/2008	Cattle	O	SEA	Mya-98	HQ116252
83	O/TAI/6/2008	Thailand	Kalasin	01/08/2008	Cattle	O	SEA	Mya-98	HQ116253

84	O/TAI/7/2008	Thailand	Mahasarakham	01/08/2008	Buffalo	O	SEA	Mya-98	HQ116254
85	O/TAI/12/2008	Thailand	Lumphun	01/11/2008	Cattle	O	SEA	Mya-98	HQ116255
86	O/TAI/1/2009	Thailand	Pathumrat, Roi Et	01/01/2009	Cattle	O	SEA	Mya-98	HQ116256
87	O/TAI/2/2009	Thailand	Chiangmai	06/01/2009	Cattle	O	SEA	Mya-98	HQ116257
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89	O/TAI/4/2009	Thailand	Nakhonpathom	01/01/2009	Pig	O	SEA	Mya-98	HQ116259
90	O/TAI/12/2009	Thailand	Jana, Songkhla	10/08/2009	Cattle	O	SEA	Mya-98	HQ116260
91	O/TAI/13/2009	Thailand	Muang, Kalasin	25/08/2009	Cattle	O	SEA	Mya-98	HQ116261
92	O/TAI/15/2009	Thailand	Watbol, Phitsan	04/09/2009	Cattle	O	SEA	Mya-98	HQ116262
93	O/TAI/16/2009	Thailand	Potharam, Ratchaburi	01/09/2009	Cattle	O	SEA	Mya-98	HQ116263
94	O/TAI/17/2009	Thailand	Khirimat, Sukhothani	02/10/2009	Cattle	O	SEA	Mya-98	HQ116264
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98	O/TAI/21/2009	Thailand	Muang, Phetchaburi	17/11/2009	Cattle	O	SEA	Mya-98	HQ116268
99	O/TAI/22/2009	Thailand	Banthi, Lumphun	18/11/2009	Pig	O	SEA	Mya-98	HQ116269
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116	O/VIT/3/2006	Vietnam	Tien Giang	12/01/2006	Pig	O	Cathay	unnamed	HQ116286
117	O/VIT/4/2006	Vietnam	Binh Dinh	08/02/2006	Pig	O	SEA	Mya-98	HQ116287
118	O/VIT/5/2006	Vietnam	Binh Dinh	17/02/2006	Cattle	O	SEA	Mya-98	HQ116288
119	O/VIT/6/2006	Vietnam	Phu Yen	01/03/2006	Pig	O	SEA	Mya-98	HQ116289
120	O/VIT/7/2006	Vietnam	Phu Yen	01/03/2006	Cattle	O	SEA	Mya-98	HQ116290
121	O/VIT/1/2008	Vietnam	Ho Chi Minh Province	01/01/2008	Pig	O	Cathay	unnamed	HQ116291
122	A/CAM/1/2006	Cambodia	-	13/07/2006	Cattle	A	Asia	unnamed	HQ116292
123	A/CAM/2/2006	Cambodia	-	13/07/2006	Cattle	A	Asia	unnamed	HQ116293
124	A/CAM/2/2008	Cambodia	Samma Kimeanchey, Kampong Chhnang	39625.0	Cattle	A	Asia	unnamed	HQ116294
125	A/LAO/4/2008	Laos	Pakson, Bolikhamxay	27/11/2008	Cattle	A	Asia	unnamed	HQ116295
126	A/MAY/1/2003	Malaysia	Tanah Merah, Kelantan	25/06/2003	Cattle	A	Asia	unnamed	HQ116296
127	A/MAY/3/2003	Malaysia	Padang Besar, Perlis	18/09/2003	Cattle	A	Asia	unnamed	HQ116297
128	A/MAY/4/2003	Malaysia	Kota Bharu, Kelantan	02/11/2003	Cattle	A	Asia	unnamed	HQ116298
129	A/MAY/5/2003	Malaysia	Kota Bharu, Kelantan	13/11/2003	Cattle	A	Asia	unnamed	HQ116299
130	A/MAY/5/2005	Malaysia	Machang, Kelantan	24/10/2005	Cattle	A	Asia	unnamed	HQ116300

131	A/MAY/1/2007	Malaysia	Tasek Gelugor, Penang	21/01/2007	Pig	A	Asia	unnamed	HQ116301
132	A/MAY/3/2007	Malaysia	Kuala Selangor, Selangor	22/07/2007	Cattle	A	Asia	unnamed	HQ116302
133	A/MAY/1/2008	Malaysia	Yan, Kedah	24/03/2008	Cattle	A	Asia	unnamed	HQ116303
134	A/MAY/3/2008	Malaysia	Kuala Langat, Selangor	18/06/2008	Cattle	A	Asia	unnamed	HQ116304
135	A/MAY/2/2009	Malaysia	Kuala Pilah, Negeri Sembilan	17/03/2009	Cattle	A	Asia	unnamed	HQ116305
136	A/MAY/9/2009	Malaysia	Ipoh, Perak	20/04/2009	Cattle	A	Asia	unnamed	HQ116306
137	A/TAI/2/98	Thailand	Loei	1998	Cattle	A	Asia	unnamed	HQ116355
138	A/TAI/1/99	Thailand	Lumphun	1999	Cattle	A	Asia	unnamed	HQ116356
139	A/TAI/6/99	Thailand	Petchaboon	1999	Cattle	A	Asia	unnamed	HQ116357
140	A/TAI/1/2001	Thailand	Loei	05/06/2001	Cattle	A	Asia	unnamed	HQ116307
141	A/TAI/3/2001	Thailand	Chiangmai	26/11/2001	Cattle	A	Asia	unnamed	HQ116308
142	A/TAI/2/2002	Thailand	Pattalung	04/03/2002	Cattle	A	Asia	unnamed	HQ116309
143	A/TAI/7/2002	Thailand	Nakhonpathom	13/03/2002	Pig	A	Asia	unnamed	HQ116310
144	A/TAI/4/2003	Thailand	Kalasin	22/07/2003	Cattle	A	Asia	unnamed	HQ116311
145	A/TAI/7/2003	Thailand	Lopburi	20/04/2003	Cattle	A	Asia	unnamed	HQ116312
146	A/TAI/8/2003	Thailand	Suphanburi	08/09/2003	Pig	A	Asia	unnamed	HQ116313
147	A/TAI/10/2003	Thailand	Yala	29/09/2003	Cattle	A	Asia	unnamed	HQ116314
148	A/TAI/11/2003	Thailand	Prachinburi	30/09/2003	Cattle	A	Asia	unnamed	HQ116315
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155	A/TAI/7/2005	Thailand	Ranong	30/11/2005	Cattle	A	Asia	unnamed	HQ116322
156	A/TAI/11/2005	Thailand	Songkhla	31/12/2005	Cattle	A	Asia	unnamed	HQ116323
157	A/TAI/1/2006	Thailand	Saraburi	16/01/2006	Cattle	A	Asia	unnamed	HQ116324
158	A/TAI/2/2006	Thailand	Lamphun	06/03/2006	Cattle	A	Asia	unnamed	HQ116325
159	A/TAI/3/2006	Thailand	Suratthani	25/05/2006	Cattle	A	Asia	unnamed	HQ116326
160	A/TAI/4/2006	Thailand	Phitsanulok	14/06/2006	Cattle	A	Asia	unnamed	HQ116327
161	A/TAI/6/2006	Thailand	Udonthani	18/09/2006	Cattle	A	Asia	unnamed	HQ116328
162	A/TAI/7/2006	Thailand	Chiangmai	25/09/2006	Cattle	A	Asia	unnamed	HQ116329
163	A/TAI/8/2006	Thailand	Nakhonpathom	07/11/2006	Cattle	A	Asia	unnamed	HQ116330
164	A/TAI/9/2006	Thailand	Nakhonratchasim	10/11/2006	Cattle	A	Asia	unnamed	HQ116331
165	A/TAI/10/2006	Thailand	Songkhla	20/11/2006	Cattle	A	Asia	unnamed	HQ116332
166	A/TAI/11/2006	Thailand	Nakhonphanom	29/12/2006	Cattle	A	Asia	unnamed	HQ116333
167	A/TAI/3/2007	Thailand	Petchaburi	02/06/2007	Cattle	A	Asia	unnamed	HQ116334
168	A/TAI/11/2007	Thailand	Songkhla	19/11/2007	Cattle	A	Asia	unnamed	HQ116335
169	A/TAI/4/2008	Thailand	Udonthani	29/07/2008	Cattle	A	Asia	unnamed	HQ116336
170	A/TAI/8/2008	Thailand	Mukdahan	01/08/2008	Cattle	A	Asia	unnamed	HQ116337
171	A/TAI/9/2008	Thailand	Mukdahan	01/08/2008	Buffalo	A	Asia	unnamed	HQ116338
172	A/TAI/10/2008	Thailand	Nongbualamphu	01/08/2008	Cattle	A	Asia	unnamed	HQ116339
173	A/TAI/11/2008	Thailand	Chiangmai	01/08/2008	Cattle	A	Asia	unnamed	HQ116340
174	A/TAI/13/2008	Thailand	Phitsanulok	11/11/2008	Cattle	A	Asia	unnamed	HQ116341
175	A/TAI/14/2008	Thailand	Songkhla	01/11/2008	Cattle	A	Asia	unnamed	HQ116342
176	A/TAI/15/2008	Thailand	Pattalung	28/11/2008	Cattle	A	Asia	unnamed	HQ116343
177	A/TAI/16/2008	Thailand	Maung, Satool	01/12/2008	Cattle	A	Asia	unnamed	HQ116344
178	A/TAI/17/2008	Thailand	Nakornsrithammarat	01/12/2008	Cattle	A	Asia	unnamed	HQ116345
179	A/TAI/18/2008	Thailand	Krabi	01/12/2008	Cattle	A	Asia	unnamed	HQ116346
180	A/TAI/19/2008	Thailand	Chiangmai	01/12/2008	Cattle	A	Asia	unnamed	HQ116347

181	A/TAI/5/2009	Thailand	Thung Song, Nakornsrithammarat	08/01/2009	Cattle	A	Asia	unnamed	HQ116348
182	A/TAI/6/2009	Thailand	Mae oon, Chiangmai	20/03/2009	Cattle	A	Asia	unnamed	HQ116349
183	A/TAI/7/2009	Thailand	Muang, Kalasin	04/02/2009	Cattle	A	Asia	unnamed	HQ116350
184	A/TAI/8/2009	Thailand	Lablae, Uttaradit	12/02/2009	Cattle	A	Asia	unnamed	HQ116351
185	A/TAI/9/2009	Thailand	Piboonruk, Udonthani	16/02/2009	Cattle	A	Asia	unnamed	HQ116352
186	A/TAI/10/2009	Thailand	Kuankanun, Pattalung	13/03/2009	Cattle	A	Asia	unnamed	HQ116353
187	A/TAI/14/2009	Thailand	Muang, Chiangmai	03/09/2009	Cattle	A	Asia	unnamed	HQ116354
188	A/VIT/4/2004	Vietnam	Ninh Thuan	15/09/2004	Cattle	A	Asia	unnamed	HQ116358
189	A/VIT/5/2004	Vietnam	Binh Dinh	15/09/2004	Cattle	A	Asia	unnamed	HQ116359
190	A/VIT/6/2004	Vietnam	Long An	22/09/2004	Cattle	A	Asia	unnamed	HQ116360
191	A/VIT/9/2004	Vietnam	Dong Nai	29/10/2004	Cattle	A	Asia	unnamed	HQ116361
192	A/VIT/10/2004	Vietnam	Binh Dinh	01/11/2004	Cattle	A	Asia	unnamed	HQ116362
193	A/VIT/11/2004	Vietnam	Tien Giang	02/11/2004	Cattle	A	Asia	unnamed	HQ116363
194	A/VIT/12/2004	Vietnam	Daklak	24/12/2004	Cattle	A	Asia	unnamed	HQ116364
195	A/VIT/8/2005	Vietnam	Dak Nong	07/05/2005	Cattle	A	Asia	unnamed	HQ116365
196	A/VIT/10/2005	Vietnam	Lam Dong	01/06/2005	Cattle	A	Asia	unnamed	HQ116366
197	A/VIT/13/2005	Vietnam	Ninh Thuan	31/07/2005	Cattle	A	Asia	unnamed	HQ116367
198	A/VIT/14/2005	Vietnam	Binh Phuoc	13/08/2005	Cattle	A	Asia	unnamed	HQ116368
199	A/VIT/18/2005	Vietnam	Binh Duong	07/12/2005	Cattle	A	Asia	unnamed	HQ116369
200	A/VIT/2/2008	Vietnam	Quang Tri	01/01/2008	Cattle	A	Asia	unnamed	HQ116370
201	A/VIT/3/2008	Vietnam	Thanh Hoa	01/01/2008	Cattle	A	Asia	unnamed	HQ116371
202	A/VIT/4/2008	Vietnam	Nghe An	01/01/2008	Buffalo	A	Asia	unnamed	HQ116372
203	A/VIT/5/2008	Vietnam	Thai Binh	01/01/2008	Cattle	A	Asia	unnamed	HQ116373
204	A/VIT/6/2008	Vietnam	Nghe An	01/01/2008	Cattle	A	Asia	unnamed	HQ116374
205	A/VIT/7/2008	Vietnam	Nghe An	01/01/2008	Buffalo	A	Asia	unnamed	HQ116375
206	A/VIT/8/2008	Vietnam	Nghe An	01/01/2008	Cattle	A	Asia	unnamed	HQ116376
207	A/VIT/1/2009	Vietnam	Thua Thien Hue	01/01/2009	Cattle	A	Asia	unnamed	HQ116377
208	A/VIT/2/2009	Vietnam	Hoa Binh	01/01/2009	Buffalo	A	Asia	unnamed	HQ116378
209	A/VIT/3/2009	Vietnam	Quang Binh	01/01/2009	Cattle	A	Asia	unnamed	HQ116379
210	A/VIT/4/2009	Vietnam	Quang Binh	01/01/2009	Cattle	A	Asia	unnamed	HQ116380
211	A/VIT/5/2009	Vietnam	Bac Kan	01/01/2009	Buffalo	A	Asia	unnamed	HQ116381
212	A/VIT/6/2009	Vietnam	Phutho	01/01/2009	Pig	A	Asia	unnamed	HQ116382
213	A/VIT/7/2009	Vietnam	Bac Kan	01/01/2009	Buffalo	A	Asia	unnamed	HQ116383
214	A/VIT/8/2009	Vietnam	San La	01/01/2009	Buffalo	A	Asia	unnamed	HQ116384

Supplementary Table S2.2: FMDV reference sequences used in this study.

No	Sample ID	Country	District/ Province	Collection date	Serotype	Topotype	Strain	Reference	Accession no
1	O/BHU/15/2003	Bhutan	NA	06/05/2003	O	ME-SA	PanAsia	Knowles et al., 2005	DQ164865
2	O/CAM/11/94	Cambodia	Mong Russey, Battambang	01/07/1994	O	SEA	Cam-94	Knowles et al., 2001	AJ294906
3	O/CAM/12/94	Cambodia	Mong Russey, Battambang	01/07/1994	O	SEA	Cam-94	Knowles et al., 2001	AJ294907
4	O/CAM/1/98	Cambodia	Kg. Speu	01/07/1998	O	SEA	Cam-94	Knowles et al., 2001	AJ294908
5	O/CAM/2/98	Cambodia	Kg. Speu	01/07/1998	O	SEA	Cam-94	Knowles et al., 2001	AJ294909
6	O/CAM/3/98	Cambodia	Kg. Speu	01/07/1998	O	SEA	Cam-94	Knowles et al., 2001	AJ294910
7	O/CAM/6/89	Cambodia	NA	01/07/1989	O	SEA	Cam-94	Knowles et al., 2001	AJ318827
8	O/CAM/2/2000	Cambodia	Angkor Chum	01/07/2000	O	ME-SA	PanAsia	Knowles et al., 2005	AJ318828
9	O/CAM/4/2000	Cambodia	Angkor Chum	01/07/2000	O	ME-SA	PanAsia	Knowles et al., 2005	AJ318829
10	O/HKN/21/70	Hong Kong	Hang Tau, Kowloon	01/07/1970	O	CATHAY		Knowles et al., 2001	AJ294911
11	O/HKN/6/83	Hong Kong	Pokfulam	01/07/1983	O	CATHAY		Knowles et al., 2001	AJ294919
13	O/LAO/4/98	Laos	Attapeu	15/09/1998	O	SEA	Cam-94	Knowles et al., 2005	DQ164906
12	O/LAO/1/98	Laos	Attapeu	15/11/1998	O	SEA	Cam-94	Khounsy et al., 2009	EU667440
16	O/LAO/2/99	Laos	Champasak	15/01/1999	O	SEA	Cam-94	Khounsy et al., 2009	EU667442
15	O/LAO/11/99	Laos	Savanakhet	15/09/1999	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667443
14	O/LAO/1/99	Laos	Savanakhet	15/12/1999	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667441
17	O/LAO/1/2000	Laos	Xayabury	15/01/2000	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667444
18	O/LAO/2/2001	Laos	Luang Nampha	15/04/2001	O	SEA	Mya-98	Khounsy et al., 2009	EU667445
19	O/LAO/4/2001	Laos	Xieng Khuang	15/08/2001	O	SEA	Mya-98	Khounsy et al., 2009	EU164907
20	O/LAO/1/2003	Laos	Xieng Khuang	15/01/2003	O	SEA	Mya-98	Khounsy et al., 2009	EU667446
23	O/LAO/3/2003	Laos	Vientiane	15/03/2003	O	ME-SA	PanAsia	Khounsy et al., 2009	DQ164908
25	O/LAO/6/2003	Laos	Luang Namtha	15/03/2003	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667447
26	O/LAO/7/2003	Laos	Vientiane	15/04/2003	O	SEA	Mya-98	Khounsy et al., 2009	EU667448
21	O/LAO/12/2003	Laos	Vientiane	15/05/2003	O	ME-SA	PanAsia	Knowles et al., 2005	DQ164910
22	O/LAO/19/2003	Laos	Bokeo	15/05/2003	O	ME-SA	PanAsia	Khounsy et al., 2009	DQ164913
24	O/LAO/35/2003	Laos	Xieng Khuang	15/12/2003	O	SEA	Mya-98	Khounsy et al., 2009	EU667450
27	O/LAO/2/2006	Laos	Bokeo	23/07/2006	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667451
28	O/LAO/3/2006	Laos	Louang Prabang	23/07/2006	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667452
29	O/LAO/4/2006	Laos	Louang Prabang	23/07/2006	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667453
30	O/LAO/5/2006	Laos	Vientiane	23/07/2006	O	ME-SA	PanAsia	Khounsy et al., 2009	EU667454

31	O/MAY/2/2000	Malaysia	Gombak, Selangor	02/02/2000	O	ME-SA	PanAsia	Knowles et al., 2005	AJ318846
32	O/MAY/3/2001	Malaysia	Kuala Langat, Selangor	23/06/2001	O	SEA	Mya-98	Knowles et al., 2005	DQ164920
33	O/MAY/5/2001	Malaysia	Tumpat, Kelantan	11/07/2001	O	SEA	Mya-98	Knowles et al., 2005	DQ164921
34	O/MAY/6/2001	Malaysia	Tumpat, Kelantan	11/07/2001	O	SEA	Mya-98	Knowles et al., 2005	DQ164922
35	O/MAY/1/2002	Malaysia	Hulu Perak, Perak	10/01/2002	O	SEA	Cam-94	Knowles et al., 2005	DQ164923
36	O/MAY/6/2003	Malaysia	Rompin, Pahang	28/12/2003	O	ME-SA	PanAsia	Schumann et al., 2008	DQ165058
38	O/MYA/1/98	Myanmar	Thar Zi, Mandalay	01/07/1998	O	SEA	Mya-98	Samuel and Knowles, 2001	AJ303521
37	O/MYA/13/89	Myanmar	NA	01/07/1989	O	SEA	Cam-94	Knowles et al., 2005	DQ164924
39	O/MYA/7/98	Myanmar	Yangon	01/07/1998	O	SEA	Mya-98	Knowles et al., 2005	DQ164925
40	O/MYA/5/99	Myanmar	Bago	01/07/1999	O	SEA	Mya-98	Knowles et al., 2005	DQ164926
41	O/MYA/2/2000	Myanmar	Yangon	23/05/2000	O	SEA	Mya-98	Knowles et al., 2005	DQ164927
42	O/MYA/7/2002	Myanmar	NA	23/10/2002	O	SEA	Mya-98	Knowles et al., 2005	DQ164928
43	O/NEP/4/2003	Nepal	NA	09/05/2003	O	ME-SA	PanAsia	Schumann et al., 2008	DQ165059
44	O/PHI/7/96	Philippines	Angono	01/07/1996	O	CATHAY		Knowles et al., 2001	AJ294926
45	O/TAI/189/87	Thailand	NA	01/07/1987	O	SEA	-	TRRL, Pakchong (unpublished)	NA
46	O/TAI/4/99	Thailand	Mae Hong Sorn	15/07/1999	O	SEA	Mya-98	Knowles et al., 2005	DQ164976
47	O/TAI/8/99	Thailand	Burirum	15/07/1999	O	SEA	Cam-94	Knowles et al., 2005	DQ164977
48	O/TAI/9/99	Thailand	Chiengrai	15/07/1999	O	ME-SA	PanAsia	Knowles et al., 2005	DQ164978
49	O/TAI/2/2000	Thailand	Songkhla	18/01/2000	O	SEA	Mya-98	Knowles et al., 2005	DQ164979
50	O/TAI/2/2003	Thailand	NA	10/03/2003	O	ME-SA	PanAsia	Knowles et al., 2005	DQ164980
51	O/TAI/3/2003	Thailand	NA	19/05/2003	O	SEA	Mya-98	Knowles et al., 2005	DQ164981
52	O/Manisa/ TUR/69	Turkey	NA	01/07/1969	O	ME-SA	-	Aktas and Samuel, 2000	AJ251477
53	O/VIT/3/97	Vietnam	NA	01/07/1997	O	CATHAY		Knowles et al., 2001	AJ294930
54	O/VIT/7/97	Vietnam	Yrongpa, Gialai	01/07/1997	O	SEA	Cam-94	Samuel and Knowles, 2001	AJ296328
55	O/VIT/10/2002	Vietnam	NA	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DG165023
56	O/VIT/12/2002	Vietnam	NA	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165024
57	O/VIT/13/2002	Vietnam	NA	14/01/2002	O	CATHAY		Knowles et al., 2005	DQ165025
58	O/VIT/14/2002	Vietnam	Kien Giang	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165026
59	O/VIT/16/2002	Vietnam	Kien Giang	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165027
60	O/VIT/19/2002	Vietnam	Kontum	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165028
61	O/VIT/20/2002	Vietnam	Binh Thuan	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DG165029
62	O/VIT/6/2002	Vietnam	NA	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165020

63	O/VIT/8/2002	Vietnam	NA	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DG165021
64	O/VIT/9/2002	Vietnam	NA	14/01/2002	O	ME-SA	PanAsia	Knowles et al., 2005	DG165022
65	O/VIT/1/2003	Vietnam	Phu Yen	15/01/2003	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165030
66	O/VIT/2/2003	Vietnam	An Giang	15/08/2003	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165031
67	O/VIT/1/2004	Vietnam	Kon Tum	15/02/2004	O	ME-SA	PanAsia	Knowles et al., 2005	DQ165032
68	O/VIT/2/2004	Vietnam	Quang Nam	15/02/2004	O	CATHAY		Knowles et al., 2005	DQ165033
69	O/VIT/3/2004	Vietnam	Quang Nam	15/03/2004	O	CATHAY		Knowles et al., 2005	DQ165034
70	A/IRN/2/87	Iran	NA	1987	A	ASIA	Iran-87	Knowles et al., 2007	EF208770
71	A/IRN/22/99	Iran	NA	1999	A	ASIA	Iran-99	Knowles et al., 2007	EF208772
72	A/IRN/1/2005	Iran	NA	2005	A	ASIA	Iran-05	Knowles et al., 2007	EF208769
73	A_{22}/IRQ/64	Iraq	NA	1964	A	ASIA	A_{22}	Carrillo et al., 2005	AY593763
74	A/LAO/36/2003	Laos	Bokeo	08/12/2003	A	ASIA		Khounsy et al., 2009	EU667455
75	A/LAO/1/2006	Laos	Vientiane	27/03/2006	A	ASIA		Khounsy et al., 2009	EU667456
76	A/LAO/6/2006	Laos	Vientiane	15/12/2006	A	ASIA		Khounsy et al., 2009	EU667457
77	A/LAO/7/2006	Laos	Vientiane	15/12/2006	A	ASIA		Khounsy et al., 2009	EU667458
78	A/LAO/8/2006	Laos	Vientiane	15/12/2006	A	ASIA		Khounsy et al., 2009	EU667459
79	A/MAY/2/2002	Malaysia	Padang Besar, Perlis	07/02/2002	A	ASIA		Schumann et al., 2008	EU414533
80	A_{15}/Bangkok/ TAI/60	Thailand	NA	1960	A	ASIA	A_{15}	Carrillo et al., 2005	AY593755
81	A/TAI/118/87	Thailand	NA	1987	A	ASIA		Knowles et al., 2007	EF208777
82	A/TAI/2/97	Thailand	NA	1997	A	ASIA		Knowles et al., 2007	EF208778
83	A/VN/02/2009	Vietnam	TT Hue	09/01/2009	A	ASIA		Le et al., 2009	GQ406248
84	A/VN/03/2009	Vietnam	TT Hue	09/01/2009	A	ASIA		Le et al., 2009	GQ406249
85	A/VN/09/2009	Vietnam	Quang Binh	06/02/2009	A	ASIA		Le et al., 2009	GQ406247
86	A/VN/11/2009	Vietnam	Quang Binh	06/02/2009	A	ASIA		Le et al., 2009	GQ406250
87	A/VN/16/2009	Vietnam	Phu Tho	17/02/2009	A	ASIA		Le et al., 2009	GQ406251
88	A/VN/20/2009	Vietnam	Bac Can	19/02/2009	A	ASIA		Le et al., 2009	GQ406252

Chapter 3

Characterisation of complete genomes of prototype foot-and-mouth disease viruses from Southeast Asia

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Some of the primers used to generate sequence data described in this chapter were designed by Müge Firat-Saraç. Complete genome sequences generated in this study have been submitted to the GenBank.

This work has been formatted for submission to: *Virus Genes*

Abstract

Foot-and-mouth disease (FMD) is endemic in mainland Southeast Asia where up to seven distinct viral lineages co-circulate. These are designated O/SEA/Mya-98, O/SEA/Cam-94, O/ME-SA/PanAsia, O/ME-SA/PanAsia-2, O/CATHAY, A/ASIA and serotype Asia 1. Distribution of outbreaks caused by these lineages is variable: O/SEA/Cam-94 has not been detected since 2003, while O/SEA/Mya-98 has been widely reported almost in all countries in the region. VP1 sequence analysis allows the temporal and geographical patterns of these FMD viruses to be defined, however complete genome sequences are required to understand the epidemiology of virus transmission at high-resolution and to investigate the processes that generate viral diversity. Therefore, the aim of this study was to generate protocols that can be used to obtain complete genome sequences for the seven FMDV lineages in Southeast Asia. Full genome sequences of the seven FMDV lineages were amplified by RT-PCR using lineage-specific primers (for the 5' untranslated region (UTR) and capsid genes) and a universal set of primers for the non-structural genes. Complete genome sequences, of various length, have been generated for each representative lineage has been generated; O/SEA/Mya-98 (8186 nt), O/SEA/Cam-94 (8160 nt), O/ME-SA/PanAsia (8190 nt), O/ME-SA/PanAsia-2 (8190), O/CATHAY (8130 nt), A/ASIA (8191 nt) and serotype Asia 1 (8176 nt). Preliminary analysis of the pandemic strain, PanAsia and its derivative, PanAsia-2, revealed interesting findings of multiple substitutions in the 5' UTR and Leader regions which suggested the possibility of recombination. These protocols will hopefully initiate further studies to define the molecular epidemiology of FMD viruses circulating in Southeast Asia.

3.1 Introduction

Foot-and-mouth disease virus (FMDV) is the prototype species of the *Aphthovirus* genus within the *Picornaviridae* family. It causes a highly contagious viral disease of cloven-hoofed animals and remains one of the most economically important diseases of domesticated livestock. The virus is antigenically highly variable and exists as seven distinct serotypes; O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2 and SAT 3 which are not uniformly distributed across the FMD endemic regions of the world (Knowles and Samuel, 2003; Rweyemamu et al., 2008). Within Southeast Asia, serotypes O and A are found in almost all mainland countries, while serotype Asia 1 has been seldom reported in recent years. Field outbreaks of FMD due to serotype C within Southeast Asia have only occurred in the Philippines and were last reported in the archipelago in 1994 (Gleeson, 2002; Rweyemamu et al., 2008).

Molecular epidemiological analyses have been widely used to understand the transboundary nature of FMDV, recreating temporal movement patterns of virus spread within countries and across international borders. Phylogenetic analysis of VP1 sequences is well documented and has been shown to be a useful tool to categorise the virus into discrete subtypes (or topotypes) which frequently show geographical clustering based on the historical distribution of the virus (Klein et al., 2006; Knowles and Samuel, 2003; Samuel and Knowles, 2001). In Southeast Asian countries, previous studies focussing on VP1 have identified up to seven FMDV lineages that are co-circulating in the region (Gleeson et al., 2003; Khounsy et al., 2009; Knowles et al., 2001a; Knowles et al., 2005). Those virus lineages were O/SEA/Mya-98, O/SEA/Cam-94, O/ME-SA/PanAsia, O/ME-SA/PanAsia-2, O/CATHAY, A/ASIA and serotype Asia 1. Prevalence of these FMDV lineages in

Southeast Asian countries varies. O/SEA/Mya-98 and A/ASIA appear to be most dominant, while viruses from the O/SEA/Cam-94 lineage were last detected in 2003. The O/SEA/Mya-98 lineage has recently caused outbreaks outside of the region in East Asian countries affecting Japan, the Republic of Korea and The Russian Federation as well as the People's Republic of China including Hong Kong Special Administrative Region (SAR) and Mongolia (Paton et al., 2010). The O/ME-SA/PanAsia, lineage that previously caused a global FMD pandemic (Knowles et al., 2005) has been reported in all Southeast Asia countries. In contrast, the O/ME-SA/PanAsia-2 lineage, which has been circulating in the Middle East and the Indian sub-continent for the last 10 years, was unique in Malaysia within Southeast Asia region.

VP1 sequence data can be used to define the relationships between viruses collected in different countries to determine the most likely origin of viruses causing field outbreaks (Abdul-Hamid et al., submitted) (Knowles et al., 2005; Konig et al., 2007). However, the resolution to which VP1 sequence data can be used to discriminate viruses is limited by the length (approx. 8% of the genome) and evolutionary rate of the VP1 gene. Furthermore, limiting sequence analysis to only VP1 reduces the ability to identify recombination events that may drive step-changes in the generation of new genetic and antigenic variants that circulate in the field (Balinda et al., 2010). Recently, full genome analysis has been used to reconstruct (at the level of farm-to-farm transmission events) the transmission histories of FMD viruses during outbreaks in the UK (Cottam et al., 2006; Cottam et al., 2008). Although complete genome sequences have been generated for a selected Southeast Asian isolates and vaccine strains in previous studies on FMDV from Thailand, Philippines and Vietnam (Carrillo et al., 2005; Le et al., 2010a; Le et al., 2010b), the

number of complete genome sequences currently available in public databases is limited. Therefore, the purpose of this study was to develop robust and reliable protocols to generate complete genome sequences of the seven FMDV lineages circulating in Southeast Asia. These tools will provide a platform for studies to investigate the molecular epidemiology of FMDV at high resolution within the region, and will hopefully enable a more comprehensive understanding of the mechanisms that generate genetic and antigenic diversity of these FMDV lineages to be elucidated.

3.2 Material and methods

3.2.1 Viruses

Cell culture supernatant for seven FMDV isolates from Malaysia were chosen representing the lineages of FMDV circulating in Southeast Asia region as previously described (Abdul-Hamid et al., submitted) (Gleeson, 2002; Khounsy et al., 2009; Knowles et al., 2005). The seven isolates (representing the different genetic lineages) selected for this study were A/MAY/3/2007 (A/ASIA), Asia1/MAY/9/1999 (Asia 1), O/MAY/7/2007 (O/SEA/Mya-98), O/MAY/7/2001 (O/SEA/Cam-94), O/MAY/3/2000 (O/ME-SA/PanAsia), O/MAY/1/2004 (O/ME-SA/PanAsia-2), and O/MAY/8/2005 (O/CATHAY). The molecular characterisation of these isolates using VP1 sequencing has been previously described (Abdul-Hamid et al., submitted) (Valarcher et al., 2009)

3.2.2 Oligonucleotide primers

Based on sequence identity between serotypes, the strategy (**Figure 3.1**) was to design universal primer sets for the non structural protein (NSP) and poly-A region and lineage-specific primers sets for the 5'-untranslated region (UTR) and

capsid coding region. Primers were designed with similar T_m (no greater than 5ºC difference across each protocol) such that a single PCR cycling programme could be used for the amplification of all the fragments across the genome. Where possible, sequence alignments (ClustalW subroutine in BioEdit sequence alignment editor v.7.0 (Hall, 1999)) used for the primer design included available sequences for FMD viruses collected from Asian countries and additional complete and partial FMDV sequences from GenBank of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

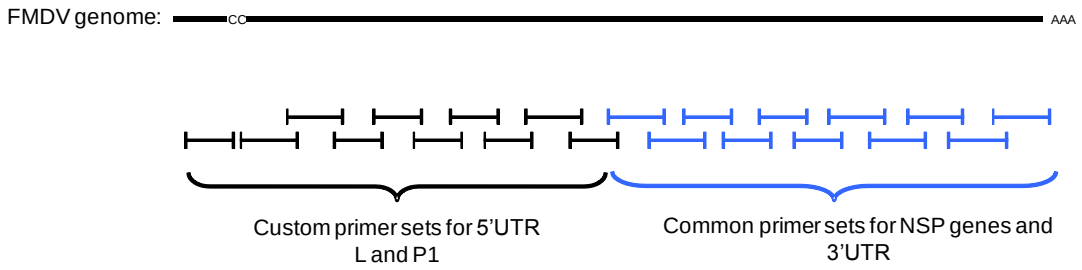

Figure 3.1: Illustration of primer design strategy for the overlapping PCR amplification of the FMDV genome.

To ensure the primers met the required criteria, readily available software such as http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/ was used for primer analysis to determine the melting temperature ($T_{m,}$), presence of secondary structure and the GC content. The PCR amplicons varied in size from 300 to 800 bp with overlaps between consecutive fragments of 50 to 200 bp. These primer sets used for each virus type are listed in **Supplementary Table S3.1a to S31.1h**. There were three alternative forward primers for the 3' UTR region.

3.2.3 RNA extraction

RNA was extracted using RNeasy® Mini Kits (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacturer's instruction. Negative control samples of nuclease-free water were processed in parallel to the test samples.

3.2.4 Two-step RT-PCR

Two-step RT-PCR was carried out as described in previously published protocols (Cottam et al., 2006) with some modifications. cDNA was prepared in three separate reactions that were combined (to provide a total cDNA volume of 120 µl) prior to PCR. These reverse transcription (RT) reactions contained 3 µl of 10 µM UKFMD Rev 6 primer (5' -GGC GGC CGC TTT TTT TTT TTT TTT-3'), 3 µl 10mM dNTP and 15 µl RNA. The tubes containing this mixture were placed in thermocycler at 70°C for 3 minutes and 4°C for 3 minutes to denature the template. RT mix was prepared in which single reactions consisted of 5x FS buffer (8 µl), 0.1mM DTT (2 µl), RNase OUT (2 µl) (Invitrogen, CA, USA), Superscript III RT (2 µl) (Invitrogen, CA, USA) and nuclease-free (NF) (5 µl). Master mix (19 µl) was then added into tube with denatured RNA and incubated in thermocycler at 45°C for 60 minutes followed by 85°C for 5 minutes and hold at 4°C. RT product was cleaned up using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). In a clean room, PCR master mix was set up for PCR according to the number of PCR primers required. The PCR mix for one reaction consisted of 10x PCR buffer (5 µl), 50mM $MgSO_4$ (2 µl), 10mM DNTP (1 µl), Platinum® High Fidelity Taq (0.25 µl) (Invitrogen) and NF water (34.76 µl) (Qiagen). Master mix (43 µl) was added into each tube followed by 2 µl of each 10 µM forward primer and 10 µM reverse primer. Three µl cDNA (or negative extraction control) was added to give final reaction volume of 50 µl. Tubes were placed in thermocycler with PCR program cycle of initial denaturation at 94°C for 5 minute, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, finishing the cycle with incubation at 72°C for 5 minutes and hold at 4° C.

The resulting PCR products were examined by electrophoresis using a 1.5% agarose gel at 100V for 40 minutes.

3.2.5 Cycle sequencing reaction

Cycle sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, Warrington, UK) using the same primers used in the PCR steps. Sequencing reactions were analysed using ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

3.2.6 Nucleotide sequence analysis

For each virus, multiple sequence read were assembled and analysed using SeqMan II (Lasergene 8.0; DNAStar Inc., WI, USA). The S and L fragments of the 5'- UTR were joined with the addition of a 10 nt artificial poly (C). The poly (A) tail was kept constant at 10 nt for the seven complete genome sequences. Sequence alignment and comparison at the nucleotide and the amino acid levels were performed in BioEdit sequence alignment editor v.7.0.5.3. Percentage identity matrices for the whole genome and complete polyprotein were determined using the DNADist and Protdist routines in BioEdit sequence alignment editor v.7.0.5.3. In addition, similarity plotting analysis was also carried out using SimPlot v. 3.5.1 (Lole et al., 1999).

3.3 Results

3.3.1 RT-PCR and sequencing

FMDV RNA was successfully amplified for the seven representative viruses of each lineage present in the Southeast Asia region. The number of PCR products used for each genome ranged between 21 to 24 fragments; 21 fragments for O/MAY/7/2001 and O/MAY/8/2005, 22 fragments for O/MAY/7/2007,

Asia1/MAY/9/99 and O/MAY/3/2000, 23 fragments for O/MAY/1/2004 and 24 fragments for A/MAY/3/2007 as shown in Figure 3.2.

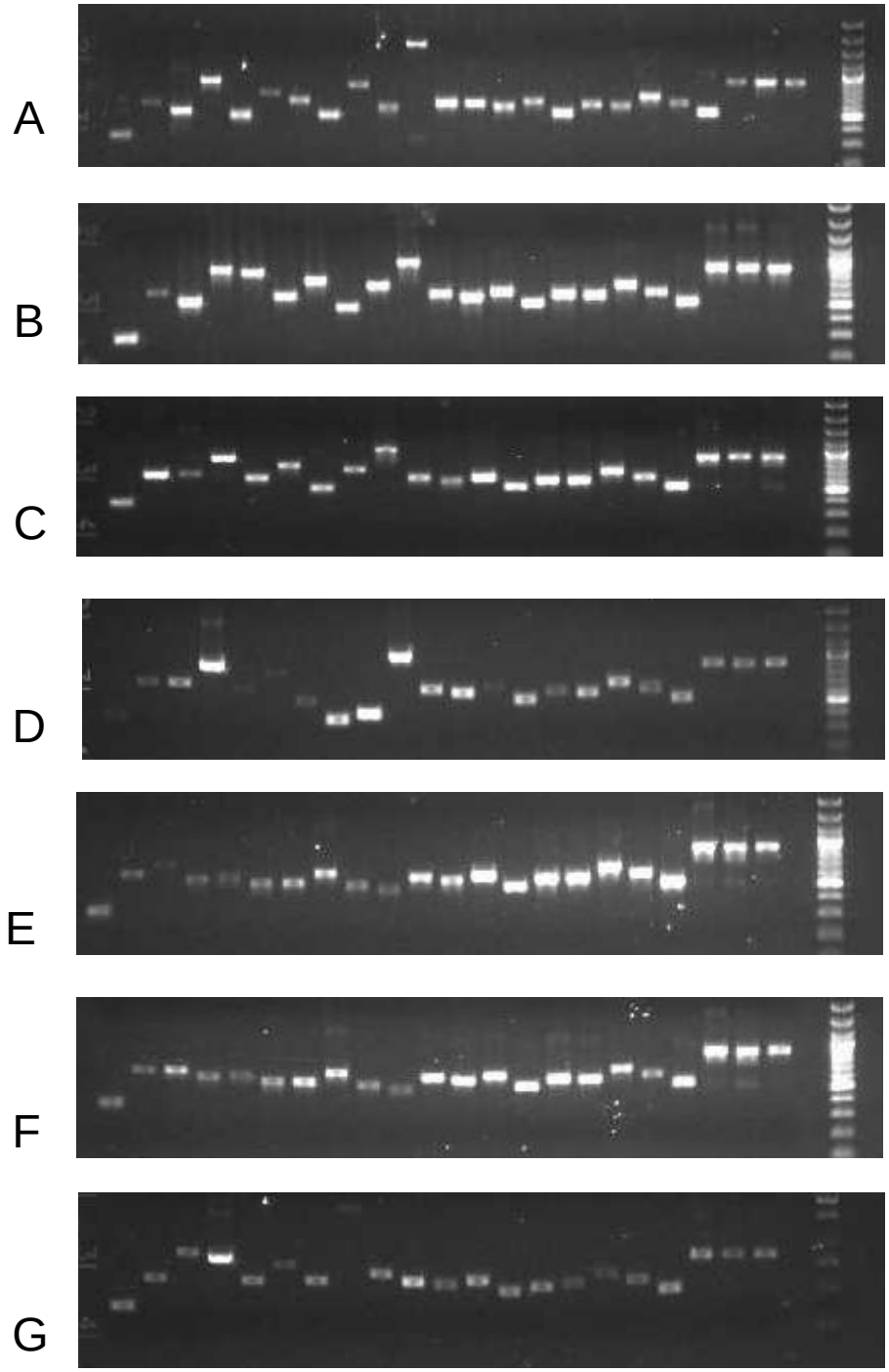

Figure 3.2: Agarose gel electrophoresis images of the component of PCR fragments for the seven representative virus lineages; [A] serotype A (A/MAY/3/2007) [B] Asia 1 (Asia1/MAY9/99) [C] O/SEA/Mya-98 (O/MAY/7/2007) [D] O/SEA/Cam-94 (O/MAY/7/2001) [E] O/ME-SA/PanAsia (O/MAY/3/2000) [F] O/ME-SA/PanAsia-2 (O/MAY/1/2004) and [G] O/CATHAY (O/MAY/8/2005).

3.3.2 Nucleotide sequence analysis

Complete genome sequences of the seven isolates were between 8130 and 8191 nucleotides in length. These sequences were aligned together with previously published sequences O/Yunlin/Taiwan/97 (AF308157), O/Chu-Pei/Taiwan/97 (AF026168) and O/South Korea/2000 (AJ539139) to identify the putative functional regions of the genome. Pairwise comparisons of the percentage nucleotide identities across the whole genome and the amino acids of the polyprotein are shown in **Table 3.1**. As expected, the two SEA topotype isolates, O/MAY/7/2007 and O/MAY/7/2001 were most closely related to each other with identities of 91.0% and 94.4% for nucleotides and amino acids respectively. The two PanAsia strains, O/MAY/1/2004 and O/MAY/3/2000 also shared a high nucleotides and amino acids identity which is 91.8% and 95.6%, respectively.

There were 647 total nucleotide changes across the genome when the two PanAsia strain viruses, O/MAY/3/2000 and O/MAY/1/2004, were compared. However, these changes were not uniformly distributed (**Figure 3.3**). The nucleotide changes were focussed in the 5' UTR and L regions of the genome with 0.133 nucleotide substitutions per site sequenced compared to 0.065 nucleotide substitutions per site sequenced at the rest of the genome region.

Table 3.1: Percent identities of nucleotides for the whole genome (blue box) and amino acid of the polyprotein (red box).

Type	Isolate	Accession no	A/MAY/3/2007	Asia1/MAY/9/99	O/MAY/7/2007	O/MAY/7/2001	O/MAY/1/2004	O/MAY/3/2000	O/MAY/8/2005
A/Asia	A/MAY/3/2007	HQ632773	ID	83.40	85.34	86.10	85.19	84.36	80.63
Asia 1	Asia1/MAY/9/99	HQ632774	85.11	ID	85.20	85.86	84.43	84.02	79.89
O/SEA/Mya-98	O/MAY/7/2007	HQ632772	84.67	85.16	ID	94.44	93.83	93.76	87.15
O/SEA/Cam-94	O/MAY/7/2001	HQ632769	86.64	87.14	90.99	ID	94.43	93.41	88.65
O/ME-SA/PanAsia-2	O/MAY/1/2004	HQ632770	84.07	83.57	88.56	89.14	ID	95.55	89.42
O/ME-SA/PanAsia	O/MAY/3/2000	HQ632768	83.45	83.44	88.85	89.04	91.82	ID	88.78
O/CATHAY	O/MAY/8/2005	HQ632771	79.32	78.91	82.57	83.44	83.68	83.28	ID

Table 3.2: The nucleotides length of the 20 fragment of the seven FMDV complete genomes.

Isolate	Type	Genome length	5'UTR				Leader	VP4	VP2	VP3	VP1
			S	Poly (C)	PK	IRES					
A/MAY/3/2007	A/Asia	8191	369	10	211	500	603	255	654	663	630
Asia1/MAY/9/99	Asia 1	8176	366	10	209	499	603	255	654	657	627
O/MAY/7/2007	O/SEA/Mya-98	8186	367	10	210	498	603	255	654	660	633
O/MAY/7/2001	O/SEA/Cam-94	8160	368	10	213	501	603	255	654	660	633
O/MAY/3/2000	O/ME-SA/PanAsia	8190	368	10	211	501	603	255	654	660	633
O/MAY/1/2004	O/ME-SA/PanAsia-2	8190	369	10	211	502	603	255	654	660	633
O/MAY/8/2005	O/CATHAY	8130	372	10	170	503	603	255	654	660	633

Isolate	Type	Genome length	2A	2B	2C	3A	3B1	3B2	3B3	3C	3D	3'UTR	Poly (A)
A/MAY/3/2007	A/Asia	8191	54	462	954	459	69	72	72	639	1410	95	10
Asia1/MAY/9/99	Asia 1	8176	54	462	954	459	69	72	72	639	1410	95	10
O/MAY/7/2007	O/SEA/Mya-98	8186	54	462	954	459	69	72	72	639	1410	95	10
O/MAY/7/2001	O/SEA/Cam-94	8160	54	462	954	426	69	72	72	639	1410	95	10
O/MAY/3/2000	O/ME-SA/PanAsia	8190	54	462	954	459	69	72	72	639	1410	94	10
O/MAY/1/2004	O/ME-SA/PanAsia-2	8190	54	462	954	459	69	72	72	639	1410	92	10
O/MAY/8/2005	O/CATHAY	8130	54	462	954	429	69	72	72	639	1410	99	10

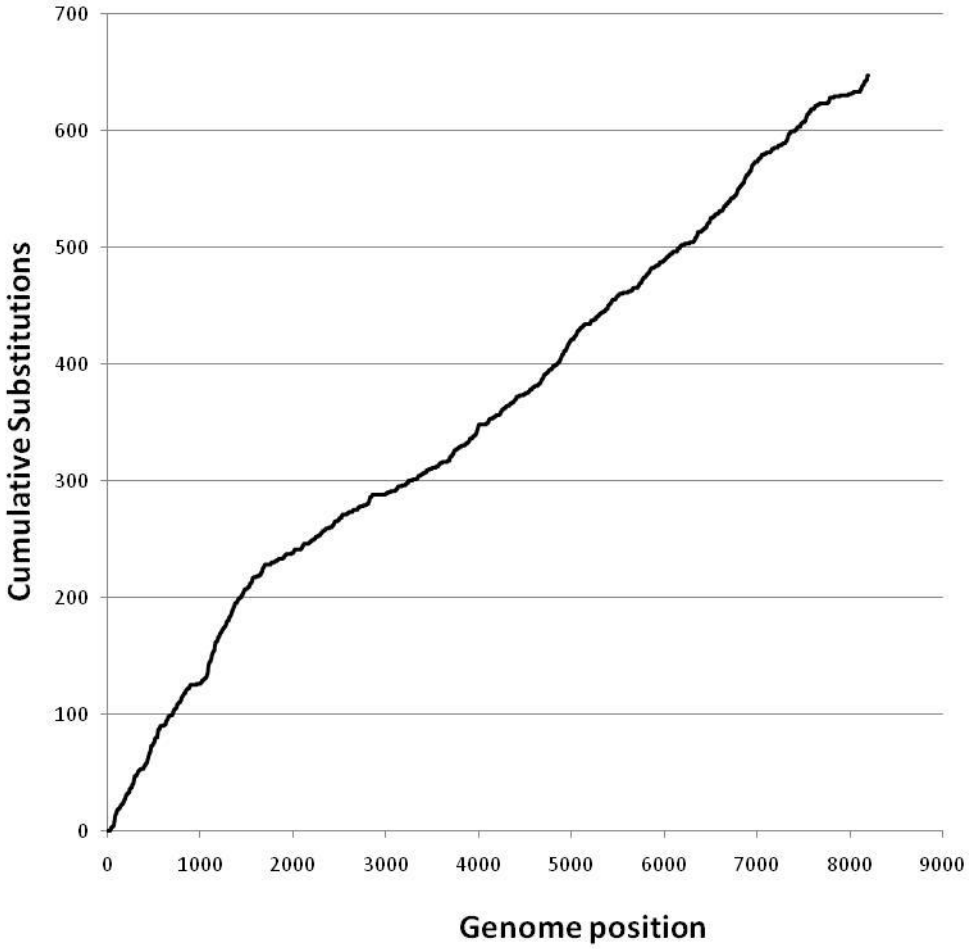

Figure 3.3: The nucleotide changes distribution of O/MAY/1/2004 when compared to O/MAY/3/2000 showing high number of changes in the first 1650 nucleotides.

FASTA analysis (http://www.ebi.ac.uk/Tools/fasta33/index.html) showed that the 5'UTR and L regions of O/MAY/3/2000 (O/ME-SA/PanAsia) were closely related to other PanAsia viruses; however, for O/MAY/1/2004 (O/ME-SAPanAsia-2), these two regions were most closely related to serotypes Asia 1 and A determined in previous studies (Carrillo et al., 2005; Mohapatra et al., 2009). BootScan and SimPlot analysis of these sequences are shown in Figure 3.4 A and B.

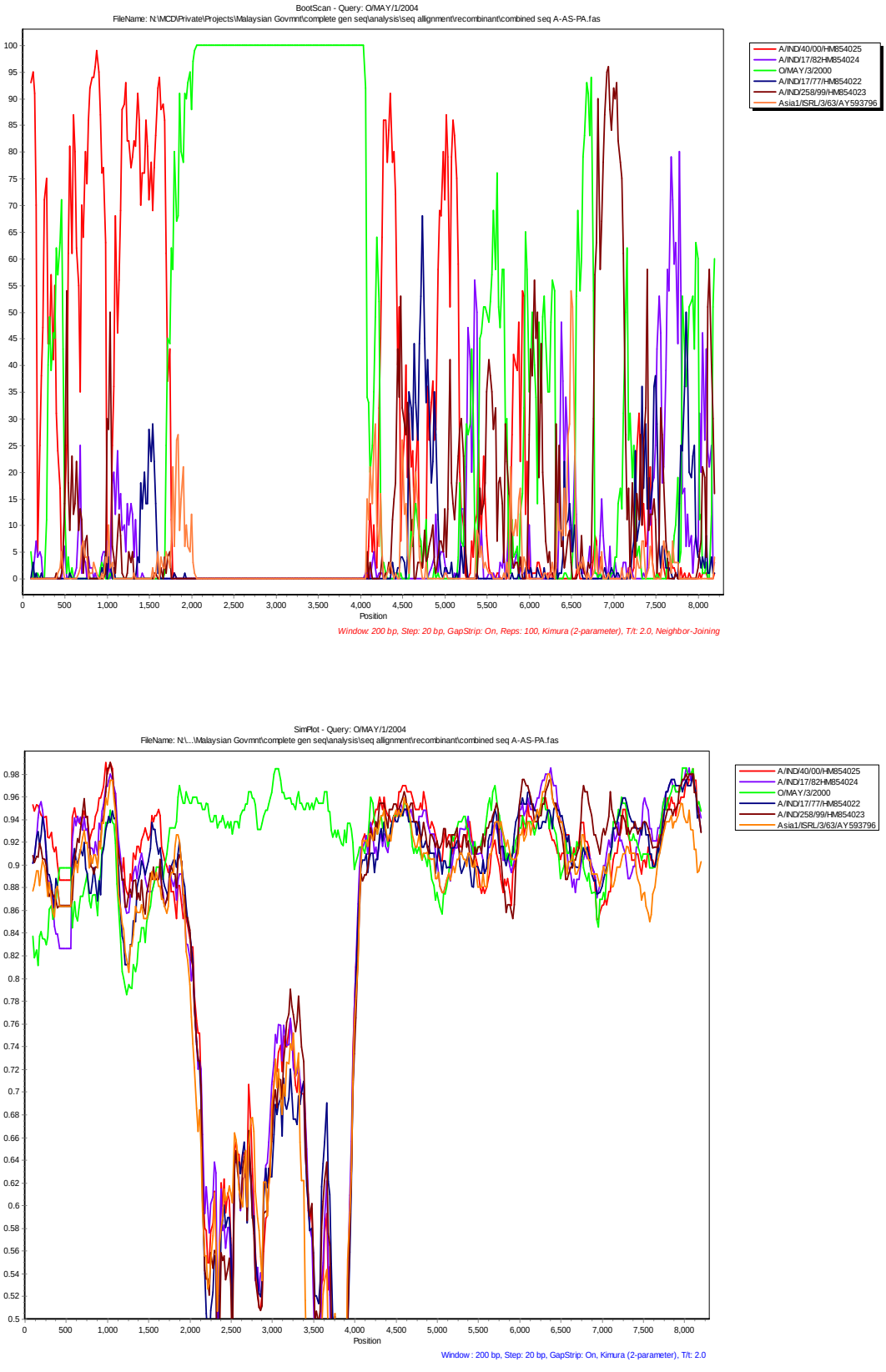

Figure 3.4: BootScan analysis (A) and SimPlot analysis (B) between O/MAY/1/2004 (PanAsia-2 virus genome) with O/MAY/3/2000 (PanAsia virus genome) and other serotypes A and Asia1 genomes.

3.3 Comparison and analysis of genomic coding region sequence

In order to undertake more detailed analysis, the whole genomes were divided into seventeen gene fragments as shown in **Table 3.2**.

5'-untranslated region

In comparison to the other FMDV genomes, the sequence for O/MAY/8/2005 had 44 nucleotide deletions which was similar to that previously seen for other CATHAY viruses (AY593835) in an earlier study (Li et al., 2007a). The AAACA sequence motif of the *cis*-acting replicative element (*cre)* structure (McKnight and Lemon, 1998) were conserved in all sequences following the poly (C) tract at nucleotide position of 619, 614, 616, 620, 619, 618 and 582 of each of the sequences; A/MAY/3/2007, Asia1/MAY/9/1999, O/MAY/7/2007, O/MAY/7/2001, O/MAY/1/2004, O/MAY/3/2000 and O/MAY/8/2005 respectively.

VP1 gene

As shown in **Table 3.2**, the VP1 genes of serotype A and serotype Asia 1 were found to be shorter than the homologous regions of serotype O viruses. Alignment of the amino acids encoded by the region revealed two amino acids deletion in these viruses immediately before the RGD motif at codon 143 and 144 of VP1 gene or at nucleotide 3681 and 3669 of the whole genome for serotype A for serotype Asia 1 respectively. In addition, an amino acid codon insertion was observed at codon 196 of VP1 gene (nucleotide 3851 of the whole genome) for serotype A. These features were common to all sequences within these lineages that are available for VP1.

3A gene

Two isolates O/MAY/7/2001 (O/SEA/Cam-94) and O/MAY/8/2005 (O/CATHAY) have deletions in the 3A region; having lengths of 426 and 429 nucleotides respectively compared to the typical length of 459 nucleotides for other sequences. The deleted region observed for the CATHAY virus was at nucleotide position 5606 to 5635 of the genome which corresponded to codon 93 to 102 of the 3A gene. The nucleotide deletion of the Cam-94 virus was observed further downstream to that of CATHAY virus at nucleotide position 5765 to 5797 of the genome which corresponded to codon 133 to 143 of the 3A gene. These finding were consistent with an earlier study on the 3A region of the porcinophilic virus and Cam-94 type viruses (Knowles et al., 2001a).

3.4 Discussion

Analysis of VP1 sequences of FMDV has provided a molecular tool to classify viruses and has allowed the tracing of outbreak origins and likely links between countries (Knowles and Samuel, 2003; Knowles et al., 2000). However, interpretation of relationships between viruses based solely on VP1 sequences has limited resolution. Recent analysis of complete genome sequences has been used for fine scale tracing of virus transmission in FMD outbreaks that occurred during 2001 and 2007 in the UK (Cottam et al., 2006; Cottam et al., 2008). In addition to improving the resolution, complete genome sequence analysis has revealed specific characteristics of FMD viruses that wouldn't be possible to infer by VP1 analysis alone (Carrillo et al., 2005). For instance, a study on the complete genome of FMD viruses collected from an epidemic in Taiwan in 1997 revealed a deletion in the 3A region which was linked to the porcinophilic phenotype of the virus (Knowles et al., 2001a).

In Asia, molecular studies to generate complete genome sequences have been carried out in South Asian and East Asian countries (Mohapatra et al., 2008; Tsai et al., 2000). However, only two previous studies have focussed on the unique lineages that circulate in Southeast Asia. This work has generated partial genome sequences for O/SEA/Mya-98, A/ASIA and serotype Asia 1 in Vietnam without the S-fragment (Le et al., 2010a; Le et al., 2010b). Therefore the purpose of this study was to generate a suite of tools suitable to generate complete genomes of virus lineages that have been reported in the Southeast Asian region. These lineages were serotype O; O/SEA/Mya-98, O/SEA/Cam-94, O/CATHAY, O/ME-SA/PanAsia and PanAsia-2, serotype A (ASIA topotype) and serotype Asia 1.

Broad-scale genome features such as block and codon deletions observed in these genomes paralleled findings for other related sequences from Southeast Asia that have been previously determined. Even though the purpose of this study was to develop technical laboratory protocols rather than to undertaken in depth genomic analysis, preliminary comparisons of the sequences generated in this study, generated an interesting finding regarding the relationship between PanAsia and PanAsia-2 viruses. The high frequency of nucleotide changes occurred in the 5'UTR and L region (222 nucleotide substitutions within 1650 nucleotide length) resulted in unequal distribution of nucleotide changes across the genome. The 5' UTR and the L^{pro} region of the PanAsia-2 virus genome were most closely related to serotype A and Asia 1 sequences, indicating that recombination may have occurred during the evolution of this lineage. Earlier studies have shown the possibility of interserotype recombination events involving the non-structural protein coding regions of FMDV genomes. Such evidence of recombination for serotype A resulted in emergence of new subtype in Iran during 2005 which was suggested to have originated from co-

infection with serotype Asia 1 (Klein et al., 2007). In addition, recombination events have also been reported between a new strain of Asia 1 serotype in China with type O PanAsia virus (Li et al., 2007b).

The protocols described in this study utilise a single set of primers for the non-structural region and lineage-specific primers for the capsid region. This method is easy to use because it applies the same primers for PCR and sequencing and uses a common RT-PCR amplification protocols for all of the PCR fragments. Seven complete genomes representing each lineage have been successfully generated. In conclusion, this method has been shown to be easy, specific and robust. The protocol has generated complete genome sequences of FMDV lineages reported in Malaysia. These sequences and the protocols will hopefully contribute in molecular epidemiology research of FMDV in the Southeast Asia region.

Acknowledgements

This study was carried out at Institute for Animal Health, Pirbright Laboratory, UK and was sponsored by Department of Public Services of the Malaysian Government and supported by the Department for Environment, Food and Rural Affairs, UK (project number SE2938) and Food and Agriculture Organisation of the United Nations (Project PR 41764).

Supplementary Tables for Chapter 3

Supplementary Table S3.1A: Primer set for non-structural protein genes

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	PCR NSP 1F	3939-3958	19	Forward	GAG ACG TYG AGT CCA ACC C	580
	PCR NSP 1R2	4517-4535	18	Reverse	CTT CTG AGG CGA TCC ATG	
2	PCR NSP 2F	4423-4443	20	Forward	CAG CTC ARA GCA CGT GAC AT	550
	PCR NSP 2R	4972-4989	17	Reverse	GCC ATR GGC GGG ATR AA	
3	PCR NSP 3F	4860-4878	18	Forward	TGA CCA CTT YGA CGG TTA	590
	PCR NSP 3R	5449-5469	20	Reverse	ACC ATC CCC TCR AAG AAY TC	
4	PCR NSP 4F	5098-5116	18	Forward	CGR AGG TTY CAC TTT GAC	500
	PCR NSP 4R	5585-5605	20	Reverse	CAT RAT CAC TAT GTT TGC CA	
5	PCR NSP 5F	5449-5469	20	Forward	GAA TTC TTT GAG GGG ATG GT	550
	PCR NSP 5R	6007-6025	18	Reverse	CAC TTT CAA AGC GAC AGG	
6	PCR NSP 6F	5831-5849	18	Forward	CRA GCT GAA GGA CCC TAC	550
	PCR NSP 6R	6377-6395	18	Reverse	GGG GGT KCC YTT CTT CAT	
7	PCR NSP 7F	6283-6302	19	Forward	GGA CAG GAC ATG CTC TCA G	640
	PCR NSP 7R	6922-6939	17	Reverse	GAC GCG TAG TCR GCA GC	
8	PCR NSP 8F	6736-6753	17	Forward	ATG CGC AAA ACC AAG CT	570
	PCR NSP 8R	7307-7324	17	Reverse	AAT TTG CGG TCC GTT GT	
9	PCR NSP 9F	7170-7189	19	Forward	RAC CTT CCT GAA GGA CGA R	500
	PCR NSP 9R	7660-7678	18	Reverse	GTC CAG CTC RAC TCC CTC	
10	PCR NSP 10F	7393-7410	17	Forward	AAC GTG TGG GAT GTG GA	832
	RACE-T21G	8186-8225	39	Reverse	CAG GAA ACA GCT ATG ACT TTT TTT TTT TTT TTT TTT TTG	
11	PCR NSP 10F	7393-7410	17	Forward	AAC GTG TGG GAT GTG GA	832
	RACE-T21C	8186-8225	39	Reverse	CAG GAA ACA GCT ATG ACT TTT TTT TTT TTT TTT TTT TTC	
12	PCR NSP 10F	7393-7410	17	Forward	AAC GTG TGG GAT GTG GA	832
	RACE-T21A	8186-8225	39	Reverse	CAG GAA ACA GCT ATG ACT TTT TTT TTT TTT TTT TTT TTA	

Supplementary Table S3.1B: Primers sets for capsid region of serotype A virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	A1F	1-19	19	Forward	TTG AAA GGG GGC GCT AGG G	372
	A1R	355-374	19	Reverse	GGG TGA AAG GCG GRC TTC G	
2	A2F3	377-397	21	Forward	CCC CCC TAA GTT TTA CCG TCR	622
	A2R	978-999	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	5UTR-2F	398-418	21	Forward	CGR CGT TAA AGG GAG GTA ACC	530
	5UTR-2R	926-945	20	Reverse	GGC ATC CTT AGC CTG TCA CC	
4	5UTR-4F	1035-1054	20	Forward	GCC TGA ATA GGY GAC CGG AG	600
	5UTR-4R	1637-1557	21	Reverse	CCG TTG AGY GGT TCT TGA TCG	
5	A4F	1296-1316	21	Forward	GAG CCT TTC TTC GAC TGG GTC	513
	A4R	1787-1809	23	Reverse	CCA TGG AGT TCT GGT ACT GRT GC	
6	A5F	1575-1593	19	Forward	GGG TGG TAR GCG ATC GAC G	714
	A5R	2272-2289	18	Reverse	WCA CCT CCA CGT CCC AGC	
7	A6F	2041-2060	20	Forward	AYT CGA GTG TGG GAG TCA CS	642
	A6R	2660-2683	24	Reverse	GCT GTT TTR GGG TCT GTT GTC ACC	
8	A7F	2492-2511	20	Forward	CTG GAC YCT GGT RGT GAT GG	501
	A7R	2975-2993	19	Reverse	GTA CGC CAC CAT GTA SCG G	
9	A8F2	2668-2687	20	Forward	GGY YTG GTG ACA ACA GAC CC	805
	A8R2	3456-3473	18	Reverse	SCG TGY TGG TGK GTT TGC	
10	A9F	3159-3176	18	Forward	GTA CAG GGY TGG GTC TGC	600
	A9R	3774-3791	18	Reverse	CGT GGC RRG AAT TGC ACC	
11	P1-3F3	3541-3557	17	Forward	CAC CTG AGG CAG CCT TG	850
	PCR NSP 1R2	4517-4532	18	Reverse	CTT CTG AGG CGA TCC ATG	
12	A10F	3545-3562	18	Forward	GGT YCC CAA TGG AGC ACC	609
	A10R	4136-4154	19	Reverse	CTT GTA CCA GGG RTT GGC C	

Supplementary Table S3.1C: Primer sets for capsid region of serotype Asia 1 virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	Asia1F	1-19	19	Forward	TTG AAA RGG GGC GCT AGG G	373
	Asia-1R	357-374	18	Reverse	GRT GAA AGG CGG GCK YCG	
2	Asia-2F	382-405	24	Forward	CCC TAA GTT TTA CCG TCG TTC CCG	622
	Asia-2R	983-1004	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	Asia-3F	865-882	18	Forward	GTG TGC AAC CCC AGC ACG	720
	Asia-3R	1567-1585	20	Reverse	CGC RTA CCA CCC DTT GGA GG	
4	Asia-4F	1430-1450	21	Forward	CYT GCT YCA CAC YGG AAT CGG	608
	Asia-4R	2019-2038	20	Reverse	GTC GTC GAY GTY GTG TGG CC	
5	Asia-5F	1803-1822	20	Forward	TCC ATG GAC ACR CAR CTT GG	624
	Asia-5R	2408-2427	20	Reverse	CCG TCA TGT TGR TGC GYG GG	
6	Asia-6F	2275-2295	21	Forward	GGG AYA TWG AGG TGA CYG CTG	604
	Asia-6R	2859-2879	21	Reverse	GGT GTT RGA CAT GTG CCC YGC	
7	Asia-7F	2729-2748	20	Forward	YGG GCG CTT CAC RAA CTT CC	603
	Asia-7R	3311-3332	22	Reverse	CCT CCG TAG TTC TCR ACY GTG G	
8	Asia-8F	3096-3116	21	Forward	CCT YTC TGC TGC WGA CTA YGC	577
	Asia-8R	3652-3673	22	Reverse	CTT CCC GTT GTA CAC TGT YGM C	
9	Asia-9F2	3536-3552	17	Forward	TGG GTG CCC AAY GGY KC	616
	Asia-9R	4133-4152	20	Reverse	AGY TTG TAC CAG GGY TTG GC	
10	P1-3F3	3541-3557	17	Forward	CAC CTG AGG CAG CCT TG	850
	PCR NSP 1R2	4517-4535	18	Reverse	CTT CTG AGG CGA TCC ATG	

Supplementary Table 3.1D: Primer set for capsid region of serotype O, Mya-98 virus.

Primer pair	Primer	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	O1F	1-24	24	Forward	TTG AAA GGG GGC GYT AGG GTY TCA	350
	O1R	354-372	19	Reverse	GGG TGA AAG GYR GGC TTY G	
2	OBFS-370F	370-386	20	Forward	CCC CCC CCC CCC CTA AG	630
	O2R2	981-1002	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	5UTR-3F	766-785	19	Forward	CGT AGC GGA GCA TGA TGG C	450
	5UTR-3R	1217-1234	17	Reverse	CRT GGT TGT TGG GYC TG	
4	UTR-573F	1092-1116	21	Forward	TTC AGG TAC CCC GAG GTA ACA	730
	VP4-73R	1792-1818	26	Reverse	GAG TTC TGG TAY TGY TGC ATR TAR TA	
5	P1-1F	1692-1708	17	Forward	GGA GCC GGG CAA TCC AG	580
	P1-1R	2262-2280	19	Reverse	CTG CRG TGA CTT CRA CGT C	
6	P1-2F	2211-2227	16	Forward	GGY GTC TAC GGC AGC C	720
	P1-2R	2937-2954	18	Reverse	CTT CGC GTC AGT GGG WCC	
7	P1-3F	2843-2860	18	Forward	GGC RGC AAA GCA CAT GTC	475
	P1-3R	3318-3335	18	Reverse	GAC CTG TGT YTC ACC RCC	
8	P1-3F2	3266-3285	20	Forward	CAC TTC GAC AGG CGA GTC AG	650
	P1-3R3	3928-3946	19	Reverse	GGG TTG GAC TCA ACG TCT C	
9	P1-3F3	3541-3557	17	Forward	CAC CTG AGG CAG CCT TG	850
	PCR NSP 1R2	4517-4535	18	Reverse	CTT CTG AGG CGA TCC ATG	

Supplementary Table S3.1E: Primer sets for capsid region of serotype O, Cam-94 virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	5UTR-1F	3-19	17	Forward	GAA AGG GGG CGC TAG GG	330
	5UTR-1R	350-367	18	Reverse	GTG AAA GGC RGG CTT CGG	
2	OP2-373F	373-392	20	Forward	CCC CCC TAA GTT TRC CGY CG	629
	O2R2	981-1002	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	5UTR-3F2	682-699	18	Forward	CCA GGT CTA GAG GRG TGA	600
	5UTR-3R2	1293-1280	18	Reverse	CGA GTC GTA GAC CCA GTC	
4	UTR-573F	1092-1116	21	Forward	TTC AGG TAC CCC GAG GTA ACA	730
	VP4-73R	1792-1818	26	Reverse	GAG TTC TGG TAY TGY TGC ATR TAR TA	
5	P1-1F	1692-1708	17	Forward	GGA GCC GGG CAA TCC AG	570
	P1-1R	2262-2280	19	Reverse	CTG CRG TGA CTT CRA CGT C	
6	P1-2F	2211-2226	16	Forward	GGY GTC TAC GGC AGC C	720
	P1-2R	2937-2954	18	Reverse	CTT CGC GTC AGT GGG WCC	
7	P1-3F	2843-2860	18	Forward	GGC RGC AAA GCA CAT GTC	475
	P1-3R	3318-3335	18	Reverse	GAC CTG TGT YTC ACC RCC	
8	P1-3F2	3266-3285	20	Forward	CAC TTC GAC AGG CGA GTC AG	350
	P1-3R2	3614-3631	18	Reverse	TGT GGT GCC GTG TAA GGC	
9	P1-3F3	3541-3557	17	Forward	CAC CTG AGG CAG CCT TG	370
	P1-3R3	3928-3946	19	Reverse	GGG TTG GAC TCA ACG TCT C	
10	P1-3F3	3541-3557	17	Forward	CAC CTG AGG CAG CCT TG	850
	PCR NSP 1R2	4517-4535	18	Reverse	CTT CTG AGG CGA TCC ATG	

Supplementary Table S3.1F: Primer sets for capsid region of serotype O, PanAsia virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	O1F	1-24	24	Forward	TTG AAA GGG GGC GYT AGG GTY TCA	371
	O1R	354-372	19	Reverse	GGG TGA AAG GYR GGC TTY G	
2	O2F	377-402	24	Forward	CCC AAG TTT TTA CCG YCT KTC CCG	625
	O2R2	981-1002	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	O3F	863-880	18	Forward	GTG TGC AAC CCC AGC ACG	718
	O3R	1564-1581	18	Reverse	CGT ACC ACC CGT TGG AGG	
4	O4F	1390-1406	17	Forward	GTG GAC CAC CCG CTC TC	581
	O4R2	1953-1971	19	Reverse	CGG TTT TCT TGT CGG CGA G	
5	O5F	1737-1758	22	Forward	CAG AAC CAR TCA GGC AAC ACT G	604
	O5R	2325-2341	17	Reverse	TCT GGC ACC ATG GCC AC	
6	O6F	2212-2234	22	Forward	CTG ACC ACA AAG GTG TCT AYG G	554
	O6R2	2744-2766	18	Reverse	GCC TCA GCC ACA TCA AGG	
7	O7F	2583-2601	19	Forward	CAC GTY GCG GGT GAG TTC C	543
	O7R	3114-3131	19	Reverse	GTC AGA CGC GGT GTA CGC	
8	O8F	2906-2924	19	Forward	CAC ACA GTA CAG CGG CAC C	636
	O8R	3523-3542	20	Reverse	CGG GAC CCA GGT RAG GTT YC	
9	O9F	3294-3310	17	Forward	GCT GAC CCC GTG ACY GC	527
	09R	3802-3821	20	Reverse	CCC TCT TCA TGC GGT AAA GC	
10	O10F	3616-3635	20	Forward	CTT GCA CTG CCT TAC ACG GC	496
	O10R	4091-4112	22	Reverse	TGA TAG CCT TCA CTC CAG TGG C	

Supplementary Table S3.1G: Primer sets for capsid region of serotype O, PanAsia-2 virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	O1F	1-24	24	Forward	TTG AAA GGG GGC GYT AGG GTY TCA	371
	O1R	354-372	19	Reverse	GGG TGA AAG GYR GGC TTY G	
2	OBFS-370F	370-386	17	Forward	CCC CCC CCC CCC CTA AG	630
	O2R2	981-1002	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	5UTR-3F2	682-699	18	Forward	CCA GGT CTA GAG GRG TGA	610
	5UTR-3R2	1293-1310	18	Reverse	CGA GTC GTA GAC CCA GTC	
4	UTR-573F	1092-1116	21	Forward	TTC AGG TAC CCC GAG GTA ACA	730
	VP4-73R	1792-1818	26	Reverse	GAG TTC TGG TAY TGY TGC ATR TAR TA	
5	O4F	1390-1406	17	Forward	GTG GAC CAC CCG CTC TC	581
	O4R2	1953-1971	19	Reverse	CGG TTT TCT TGT CGG CGA G	
6	O5F	1737-1758	22	Forward	CAG AAC CAR TCA GGC AAC ACT G	604
	O5R	2325-2341	17	Reverse	TCT GGC ACC ATG GCC AC	
7	O6F	2212-2234	22	Forward	CTG ACC ACA AAG GTG TCT AYG G	554
	O6R2	2744-2766	18	Reverse	GCC TCA GCC ACA TCA AGG	
8	O7F	2583-2601	19	Forward	CAC GTY GCG GGT GAG TTC C	543
	O7R	3114-3131	19	Reverse	GTC AGA CGC GGT GTA CGC	
9	O8F	2906-2924	19	Forward	CAC ACA GTA CAG CGG CAC C	636
	O8R	3523-3542	20	Reverse	CGG GAC CCA GGT RAG GTT YC	
10	O9F	3294-3310	17	Forward	GCT GAC CCC GTG ACY GC	527
	09R	3802-3821	20	Reverse	CCC TCT TCA TGC GGT AAA GC	
11	O10F	3616-3635	20	Forward	CTT GCA CTG CCT TAC ACG GC	496
	O10R	4091-4112	22	Reverse	TGA TAG CCT TCA CTC CAG TGG C	

Supplementary Table S3.1H: Primer sets for capsid region of serotype O, CATHAY virus.

Primer pair	Primers	Position	Primer length	Forward/ Reverse	5'-3' sequence	Amplicon size, bp
1	5UTR-1F	3-19	17	Forward	GAA AGG GGG CGC TAG GG	330
	5UTR-1R	350-367	18	Reverse	GTG AAA GGC RGG CTT CGG	
2	OBFS-370F	370-386	17	Forward	CCC CCC CCC CCC CTA AG	600
	O2R2	981-1002	22	Reverse	CCT TCT CAG ATC CCG AGT GTC G	
3	5UTR-4F	1035-1055	20	Forward	GCC TGA ATA GGY GAC CGG AG	600
	5UTR-4R	1637-1658	21	Reverse	CCG TTG AGY GGT TCT TGA TCG	
4	UTR-573F	1092-1116	21	Forward	TTC AGG TAC CCC GAG GTA ACA	730
	VP4-73R	1792-1818	26	Reverse	GAG TTC TGG TAY TGY TGC ATR TAR TA	
5	P1-1F	1692-1709	17	Forward	GGA GCC GGG CAA TCC AG	570
	P1-1R	2262-2281	19	Reverse	CTG CRG TGA CTT CRA CGT C	
6	P1-2F	2211-2227	16	Forward	GGY GTC TAC GGC AGC C	720
	P1-2R	2937-2955	18	Reverse	CTT CGC GTC AGT GGG WCC	
7	P1-3F	2843-2861	18	Forward	GGC RGC AAA GCA CAT GTC	500
	P1-3R	3318-3336	18	Reverse	GAC CTG TGT YTC ACC RCC	
8	P1-3F2	3266-3286	20	Forward	CAC TTC GAC AGG CGA GTC AG	650
	P1-3R3	3928-3947	19	Reverse	GGG TTG GAC TCA ACG TCT C	
9	P14F	3316-3338	22	Forward	CGGACATTGCGTTCATACTAGA	600
	P14R	3975-3996	21	Reverse	TCTGGTTGACAGTGTCCACCA	

Chapter 4

Full genome sequence analysis of foot-and-mouth viruses recovered from recent field outbreaks in Hong Kong SAR reveal a block deletion that maintains the RNA stem-loop of the S-fragment

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This work has been formatted for submission as a short communication to: *Virus Research*

Abstract

During 2010, a series of serotype O foot-and-mouth disease (FMD) outbreaks have occurred in East Asia affecting the People's Republic of China including Hong Kong Special Administrative Region (SAR), Republic of Korea, Japan, Mongolia and the Russian Federation. Some of these countries have been free from FMD for nearly ten years. The FMD virus responsible for this new incursion has been characterised as belonging to the Mya-98 strain of the Southeast Asia (SEA) topotype, a lineage normally restricted to countries of mainland Southeast Asia. In this study, two complete genome sequences were generated for representative viruses from Hong Kong, SAR (O/HKN/20/2010) and the Republic of Korea (O/SKR/4/2010). These complete genomes were 8123 and 8190 nucleotides in length respectively and shared nucleotide identity > 98%. There were 94 nucleotide substitutions observed across the genomes with one amino acid codon insertion for O/HKN/20/2010 and one amino acid codon deletion for O/SKR/4/2010 in the L^{pro} gene. In addition, comparison between these sequences revealed a deletion of 70 nucleotides within the 5'-untranslated region for the Hong Kong isolate which maintained a shorter RNA stem-loop that has been predicted for the S-fragment. Similar deletions were also evident in a further five related samples collected during 2010. These complete genome sequences and protocols used to generate them will contribute to further studies that are required to define the regional epidemiology of these recent FMD outbreaks.

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animal such as cattle, buffalo, pigs and small ruminants (Domingo et al., 2002). It is caused by FMD virus (FMDV) which classified in the *Aphthovirus* genus within the *Picornaviridae* family. The virus is grouped into seven distinct serotypes; O, A, C, Asia 1, SAT (Southern African Territories) 1, SAT 2 and SAT 3 which are supported by genetic sequencing of the outer capsid-coding region, e.g. VP1 sequence data (Knowles and Samuel, 2003; Samuel and Knowles, 2001). FMD incursion into disease-free countries can have a devastating economic impact as was shown in Taiwan during 1997 when an FMD epidemic in pigs was estimated to have cost approximately USD1.6 billion to control (Yang et al., 1999). Countries in East Asia such as Japan and the Republic of Korea have regained their FMD-free status without vaccination since the last FMD outbreak caused by the PanAsia strain of serotype O in 2000 (Knowles et al., 2005; Sakamoto and Yoshida, 2002).

Recently, in early 2010, FMD outbreaks have occurred in East Asia affecting the People's Republic of China including Hong Kong Special Administrative Region (SAR), Republic of Korea, Japan, Mongolia and the Russian Federation (Paton et al., 2010). From April to May 2010, epithelium tissue samples from outbreaks in Hong Kong and the Republic of Korea were received at World Reference Laboratory for FMD (WRLFMD), Pirbright for virus diagnosis and characterisation. Viruses were isolated from clinical samples in primary bovine thyroid cultures (BTy) and renal swine cell line (RS-2) and identified as serotype O by antigen typing ELISA (Ferris and Dawson, 1988). Phylogenetic analysis of VP1 characterised these viruses and sequences received from other outbreaks in the region into Mya-98 strain of Southeast Asia (SEA) topotype. This interesting virus lineage is normally restricted to countries in mainland Southeast Asia countries such as Thailand, Cambodia, Lao

People's Democratic Republic, Myanmar, Vietnam and Malaysia (Knowles and Samuel, 2003).

In order to fully characterise these new emerging viruses, complete genome analyses of two isolates, O/HKN/20/20101 from Hong Kong SAR and O/SKR/4/2010 from Republic of Korea was undertaken utilising a method that was initially developed to sequence related FMDV serotype O/SEA/Mya-98 viruses from Southeast Asian countries. This protocol amplifies 22 overlapping PCR fragments spanning the whole genome; the first 10 primer sets that are lineage-specific cover the 5' -untranslated region (UTR) and capsid genes, while the remaining 12 primer sets are designed to amplify the non-structural genes and the 3' -UTR region for all viruses from Southeast Asia (Abdul-Hamid et al., submitted).

Briefly, total RNA was extracted from antigen grown in BTy cells using RNeasy® Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) according to the manufacturer's instructions. cDNA was synthesized using UKFMD Rev 6 primer (5'-GGC GGC CGC TTT TTT TTT TTT TTT-3') (Cottam et al., 2006) and SuperScript™ III Reverse Transcriptase (Invitrogen, CA, USA). The viral cDNA was amplified using Platinum® High Fidelity Taq (Invitrogen, CA, USA) to generate 22 PCR fragments that ranged in from 300 to 700 base pairs and with each other overlapped by 100 to 200 base pairs. The PCR products (**Figure 4.1**) were analysed by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide and purified using Illustra™ GFX PCR DNA and Gel Band Purification Kit as instructed by the manufacturer. Sequencing was carried out using using BigDye® Terminator v3.1 in ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA) with the individual respective primers used for the PCR step. Complete genomes of the

two samples were assembled using SeqMan II (Lasergene 8.0; DNAStar Inc., WI, USA) generating contigs of 8123 and 8190 nucleotides for O/HKN/20/2010 and O/SKR/4/2010, respectively. Poly (C) and poly (A) tracts were kept at constant length of 10 nucleotides for each genome. The complete genome sequences were compared and aligned with partial genome sequences from Vietnam that are available for the Mya-98 lineage (GenBank accession numbers: GU125650, GU125648, GU125647 and GU125649) (Le et al., 2010b) using the ClustalW subroutine in BioEdit sequence alignment editor v.7.0.5.3 (Hall, 1999). In addition, one complete genome sequence (O/MAY/7/2007: Abdul-Hamid et al., in prep) of the same lineage generated for an isolate collected in Malaysia was included in the analyses.

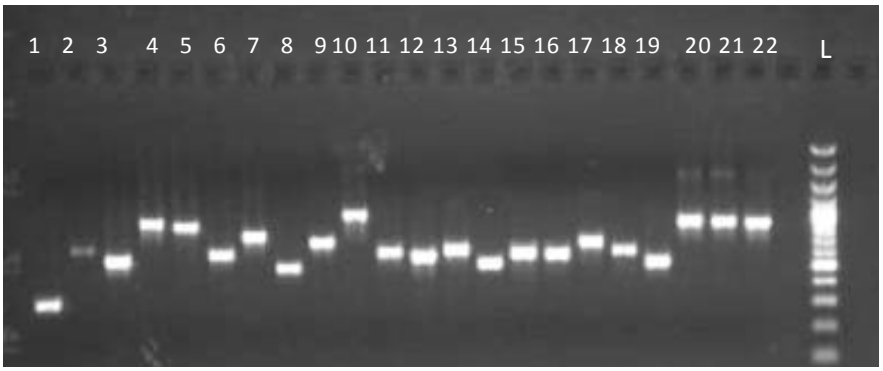

Figure 4.1: Agarose gel electrophoresis images of the component of 22 PCR fragments of O/HKN/20/2010.

Pairwise comparison between the two sequences from Hong Kong SAR and Republic of Korea demonstrated high nucleotide identities across the genome: values for L^{pro} and polyprotein regions (P1, P2 and P3) were 97.6%, 98.6%, 99.5% and 99.0%, respectively. There were only 94 nucleotides substitutions randomly distributed across the genomes. In contrast, both viruses shared lower identities (<94%) to the genome sequences for viruses collected in Southeast Asia. Translation

of these sequences highlighted an amino acid insertion within L^{pro} of O/HKN/20/2010 at codon 10 (position 1046 of the genome) while an amino acid deletion was observed for O/SKR/4/2010 at codon 28 (position 1171 of the genome).

The sequences alignment also revealed that O/HKN/20/2010 has a 70 nucleotide deletion that corresponds to positions 149 to 218 of O/SKR/4/2010. To ensure that this deletion was not an artefact of the RT-PCR procedure or due to the passage of virus in tissue culture, cDNA was prepared from clinical samples (epithelium tissue samples) from related isolates from Hong Kong SAR and one further sample from Thailand (O/TAI/22/2009) and subjected to RT-PCR and sequencing in the same region. In addition, a new forward primer (O1F2: 5'-ACC GAC TAG TAC TCT TAA CAC TCC GC-3') was designed to target the deleted region, paired with the original reverse prime, O1R and tested with the same cDNA samples. Previous analysis of the VP1 genes of the viruses from Hong Kong SAR showed that they were clustered into two distinct groups (**Figure 4.2**: paper in prep. - details available at http://www.wrlfmd.org/fmd_genotyping/asia/hkn.htm).

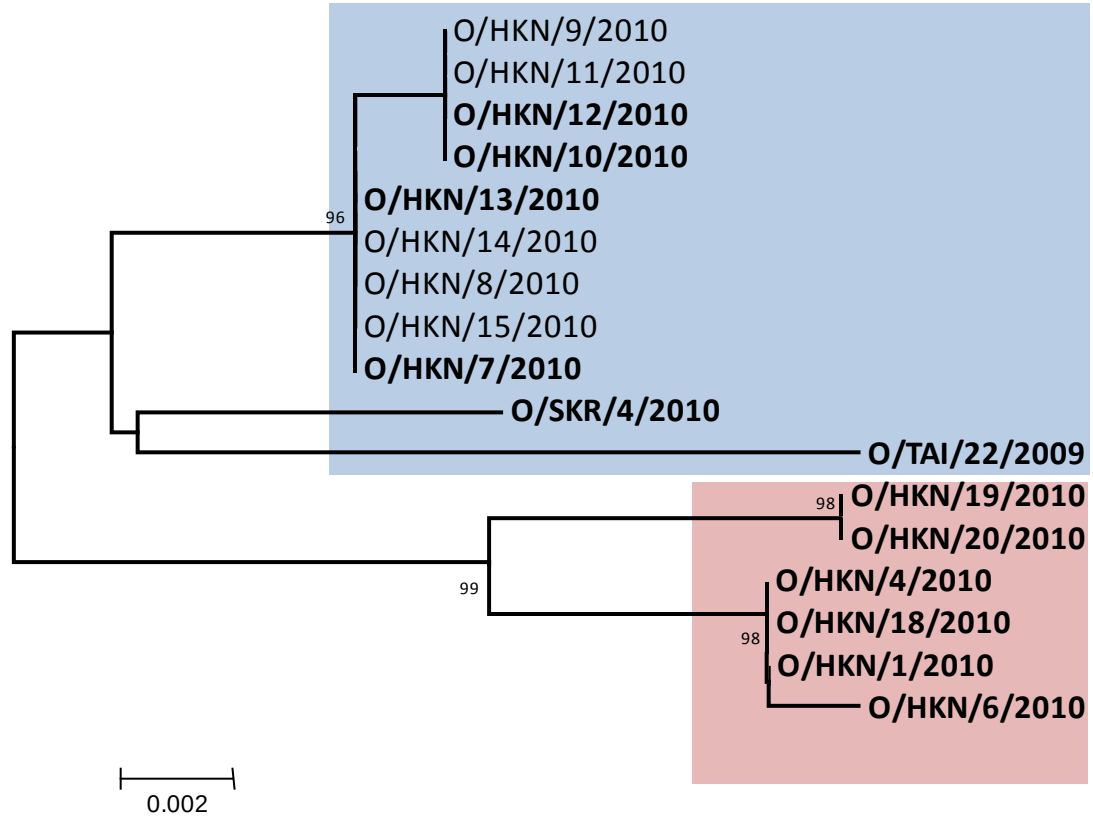

Figure 4.2: Midpoint-rooted Neighbor-joining phylogenetic tree of Hong Kong viruses with viruses from Republic of Korea and Thailand. Isolates in bold are those for which the S-fragments have been sequenced. The blue box indicates isolates without the 70 nt deletion while the red box indicates isolates with the deletion within the S-fragment.

Viruses that clustered together with O/HKN/20/2010 generated PCR products of 300 bp corresponding to identical sequence deletions in the S-fragment, whereas isolates that were more distantly related did not have a deletion as shown by a PCR fragment size of 370 bp (**Figure 4.3A**). Furthermore samples that contained the deletion were negative with the O1F2 primer (**Figure 4.3B**). Sequence data S-fragment of isolates in **Figure 4.3** with additional isolates shown in **Figure 4.2** confirmed these results (**Figure 4.4**), and for O/HKN/20/2010 identical sequences were obtained for the epithelium tissue and the tissue culture isolate.

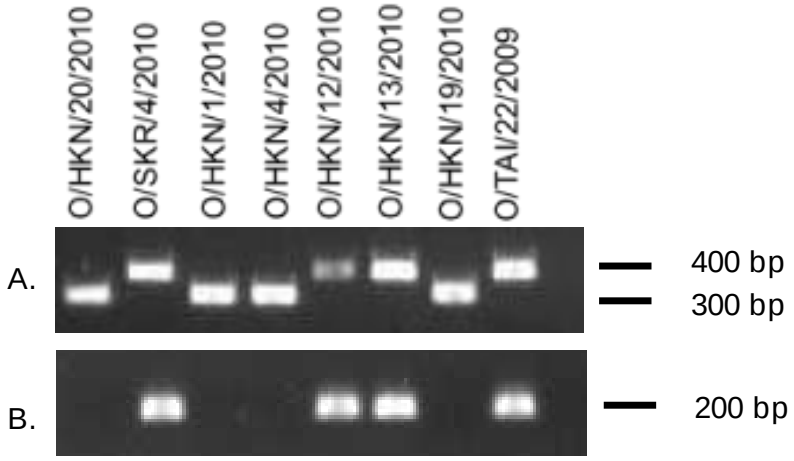

Figure 4.3: Agarose gel showing amplification of S-fragment for O/HKN/20/2010 and the other isolate from the same lineage: (a) Two different product size using original primer set O1F-O1R; 370 bp showed by isolate without deletion and 300 bp showed by isolate with deletion. (b) PCR product using the new forward primer O1F2, only isolates without deletion produce band sized 200 bp.

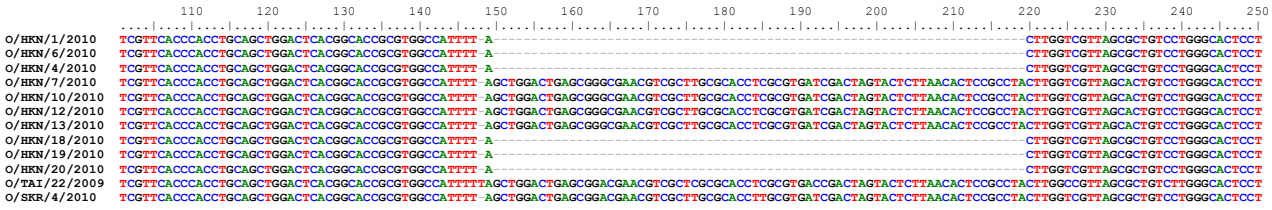

Figure 4.4: Alignment of part of S-fragment sequences showing a block deletion region of Hong Kong's viruses.

The RNA sequence of the S-fragment is predicted to fold into a long stem-loop (Clarke et al., 1987; Newton et al., 1985) which was reconstructed for O/HKN/20/2010, O/SKR/4/2010 and O/TAI/22/2009 using RNAStructure v 3.5 (Mathews, 2006) and RNAdraw version 1.1 (Matzura and Wennborg, 1996). Despite of having a block deletion, the predicted secondary structure of O/HKN/20/2010 conserved this single stem loop. The sequence deletion impacted only upon the apex of the stem-loop generating a structure that was 35 pairs shorter than for the other viruses (**Figure 4.5**). The fact that these structures appear to be highly conserved in

the face of genome deletions provides further support for the presence of this predicted single stem-loop with the S-fragment.

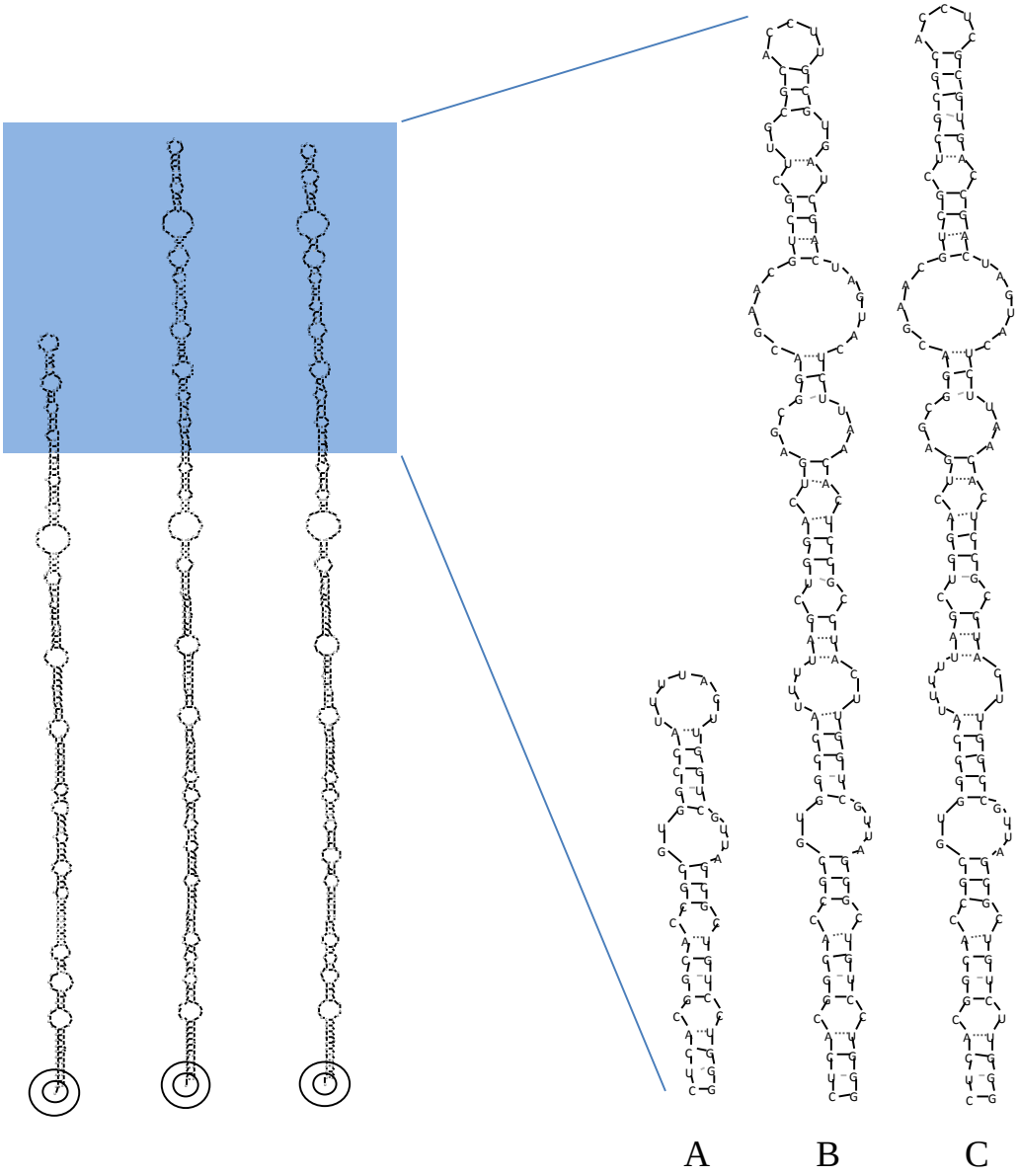

Figure 4.5: Predicted secondary structure of the S-fragment of A) O/HKN/20/2010 showed significant shorter length at the apex of the region compared to S-fragment of B) O/SKR/4/2010 and C) O/TAI/22/2009.

This finding of an unexpected deletion within the S-fragment of O/HKN/20/2010 has not been observed previously for any serotype O sequence or for any FMD virus in Asia. However, previous comparative genomic studies of FMDV (Carrillo et al., 2005) have highlighted two instances of similar deletions for serotype A isolates from Argentina in 1959 and 1961 (AY593769 and AY593789) and one additional serotype C isolate from the UK in 1934 (AY593810). Interestingly, when these sequences were aligned, the deletions were shown to occur at exactly at the same location within the S-fragment. These features do not appear to be species dependent, since they have been observed in viruses recovered from both pigs (East Asia) and cattle (South America and Europe). Earlier studies have suggested that the S-fragment plays a role in viral replication, as well as contributing to pathogenesis (Mason et al., 2003a). Further studies are required to determine whether these changes impact upon the viral phenotype.

In this study we have shown that a complete genome sequencing protocol developed for the O/SEA/Mya98 lineage mainland Southeast Asia (Abdul-Hamid et al., in prep) can be used to sequence viruses recovered from recent field outbreaks in East Asia. This protocol is particularly suited to undertake high resolution molecular analysis of viruses from the East Asia and Southeast Asia region. Similar approaches have been used to characterise new virus strains, such as the pandemic strain, PanAsia (Mason et al., 2003b), and has revealed unique characteristics of genome sequences such as those shown for the porcinophilic CATHAY topotype (Knowles et al., 2001a), as well as mechanisms that generate viral diversity such as interserotypic or intraserotypic recombination (Lee et al., 2009). These data can also be used to reconstruct transmission routes within field outbreaks of FMD as demonstrated in previous study (Cottam et al., 2006; Cottam et al., 2008). Therefore

this protocol can be applied to map viruses collected from these recent outbreaks and to formally define the possible virus transmission routes between countries affected by the recent outbreaks in the East Asia.

Acknowledgements:

Protocol development was funded by MSc studentship awarded to F. Hamid from the Malaysian Government and was supported by UK Defra (project SE2938) and FAO/EuFMD (Project PR 41764) and for funding and support. We would also like to thank our colleagues in the Republic of Korea and Hong Kong SAR for providing the samples. The authors would also like to thank Müge Firat-Sarac and Jemma Wadsworth for help and assistance, particularly in sequencing and primer design.

Archive Number 20100514.1578
Published Date 14-MAY-2010
Subject PRO/AH> Foot & mouth disease - Japan (08): (MZ) update, genome sequence

FOOT AND MOUTH DISEASE - JAPAN (08): (MIYAZAKI) UPDATE, GENOME SEQUENCE
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A ProMED-mail post
<http://www.promedmail.org>
ProMED-mail is a program of the
International Society for Infectious Diseases
<http://www.isid.org>

In this update:
[3] A full genome sequence
Date: Fri 14 May 2010
From: Donald king (IAH-P)
<donald.king@bbsrc.ac.uk>

Foot & mouth disease - Far East (P.R. China [including Hong Kong SAR], Republic of Korea and Japan)

The FAO/OIE Reference Laboratory for FMD at the Institute for Animal Health (UK) have determined the full genome sequence for a representative serotype O isolate recovered from the recent outbreaks of FMD that have occurred in the Far East. Since February this year, outbreaks due to the southeast Asia topotype (Mya-98 lineage) of serotype O have been confirmed in PR China (including Hong Kong SAR), Republic of Korea and Japan. VP1 sequence analysis undertaken at the IAH shows that viruses from Japan, Republic of Korea and Hong Kong SAR are closely related to each other (sharing nucleotide identities >97 percent;
<http://www.wrlfmd.org/fmd_genotyping/>).

A full genome sequence (8123 nucleotides) was generated from an isolate obtained from a pig with epithelial lesions that was sampled in Hong Kong SAR on 3 Mar 2010. This sequence (draft GenBank submission) is attached to the end of this bulletin or can be found at <http://www.wrlfmd.org/fmd_genotyping/far_east_2010.htm>.
[Unfortunately, ProMED-mail is technically unable to include the full genome sequence in this posting; subscribers are, therefore, referred to the URL, kindly provided by the authors, where the draft sequence can be downloaded. The URL includes also a short overview "FMD Situation in the Far East - 2010". - Mod.AS]

During previous FMD outbreaks in the UK (2001 and 2007), full genome sequence data has been used to reconstruct transmission pathways at the farm-to-farm level: for examples see Cottam et al., 2006, and Cottam et al., 2008. This approach could be used now to support epidemiological investigations that are being conducted to understand the regional and local spread of the virus in Asia.

This sequence can also be used to check the suitability of molecular tests used for the diagnosis of FMD in the region.

Further information, including detailed laboratory RT-PCR protocols used to generate this sequence, can be obtained from WRLFMD:
<faizah.hamid@bbsrc.ac.uk>, <jef.hammond@bbsrc.ac.uk> or
<donald.king@bbsrc.ac.uk>.

References:
Cottam et al., (2006) Molecular Epidemiology of Foot-and-mouth disease virus outbreak in the United Kingdom. J. Virol. 80: 11274.
Cottam et al., (2008) Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathogens 4: e1000050.

[Byline: Nor Faizah Abdul Hamid, Jemma Wadsworth, Nick Knowles, Jef Hammond and Donald King, OIE/FAO Reference Laboratory for FMD (WRLFMD) Institute for Animal Health Pirbright United Kingdom]

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[**The authors are gratefully acknowledged for the above 1st-hand information, an exemplary expression of international scientific cooperation. Hopefully, this will lead to the elucidation of the epidemiological history of the epizootic and assist in its early eradication. - Mod.AS**]

[see also:
Foot & mouth disease - Japan (07): (MZ) update 20100513.1563
Foot & mouth disease - Japan (06): (MZ) update 20100511.1536
Foot & mouth disease - Japan (05): (MZ) update 20100509.1520
Foot & mouth disease - Japan (04): (MZ) serotype O, gene 20100507.1487
Foot & mouth disease - Mongolia: (DD) bovine, OIE 20100506.1474
Foot & mouth disease - Japan (03): (MZ) update 20100505.1463
Foot & mouth disease - Japan (02): (MZ) update, RFI 20100502.1429
Foot & mouth disease - Japan: OIE report 20000520.0793]

Supplementary information for Chapter 4: letter to Promed outlining full genome sequence data for virus from Hong Kong (O/HKN/20/2010)

Chapter 5

Discussion and conclusion

5.1 Overview of the research

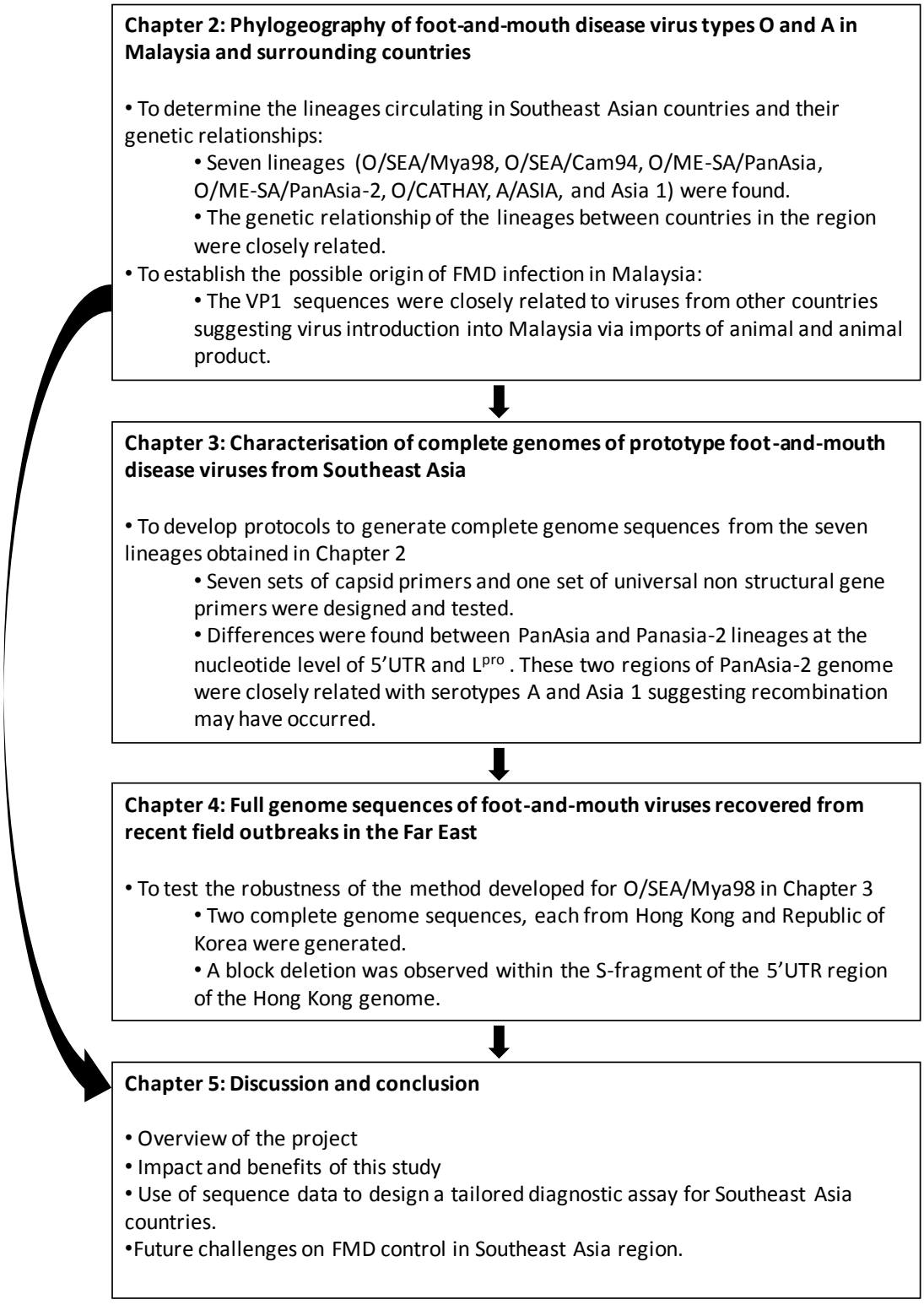

Figure 5.1: Summary of research undertaken in this study

The initial step of this study was to characterise the FMDV lineages that circulate in Malaysia and other neighbouring countries in the Southeast Asia region. Therefore, a comprehensive study of VP1 phylogentic analysis was carried out on 214 sequences from Southeast Asian countries obtained from FMD outbreaks between 2000 to 2009. This study has proven the presence of multiple lineages (seven lineages) co-circulating in the Southeast Asia region with evidence of FMDV spread between countries. These lineages were O/SEA/Mya98, O/SEA/Cam94, O/ME-SA/PanAsia, O/ME-SA/PanAsia2, O/CATHAY, serotype A (ASIA) and Asia 1. In order to understand the molecular epidemiology of FMDV in the region at higher resolution, specific protocols to generate complete genomes sequences of each lineage were developed. Recent FMD outbreaks in East Asian countries caused by the O/SEA/Mya-98 lineage which is normally reported in Southeast Asian countries, (China, Republic of Korea, Japan, Mongolia and the Russian Federation), has given the opportunity to use the lineage specific protocol to generate complete genome sequences. Two complete genome sequences, each from Hong Kong SAR and Republic of Korea were generated with an interesting finding of a block deletion within the S-fragment of the Hong Kong virus.

5.2 Impact and benefits of the study

This study has showed the importance of molecular diagnostic tools such as VP1 sequencing which are not currently available in most of Southeast Asian countries particularly Malaysia. Knowing the lineages responsible for FMD outbreaks and their possible origin will provide useful information for the epidemiological investigation of outbreaks and could become a basis for immediate disease control in Malaysia or in the neighbouring countries. In terms of methods

currently used in the region to type viruses, conventional approaches such as antigen detection ELISA with polyclonal antisera may not be sufficient to fully characterise viruses causing field outbreaks. This limitation is especially relevant for serotype O viruses since the multiple lineages (ME-SA, SEA and CATHAY) that are responsible for the outbreaks in Southeast Asian countries cannot be differentiated using existing sandwich immunoassays.

While VP1 sequences can define the FMDV lineages that are causing the outbreaks in the field, higher resolution molecular analysis may be required in order to understand the complex epidemiology of FMDV at fine scales. In addition, complete genome sequences analysis can become a tool to understand the mechanism that generate genetic and antigenic diversity such as recombination as indicated in this study when the PanAsia and PanAsia-2 genomes were compared. These protocols to generate complete genome sequences have shown to be robust and one of the methods was used to obtain sequences from viruses recovered from recent outbreaks in East Asia. The emergence of O/SEA/Mya-98 outside of the geographic region where it is normally present parallels the pandemic spread of FMD in 2000 to 2005 which was caused by the PanAsia strain. The protocols developed in this study can provide a fine scale epidemiology study to allow a reconstruction of the FMDV transmission pathway that has caused the emergence of this lineage in countries across East Asia. This approach would enable investigators to trace back the source of the infection which will be essential to achieve efficient regional disease control. In addition, with the availability of these protocols would hopefully initiate in depth molecular epidemiology study for a more comprehensive understanding of the diversity and evolution of FMDV in Southeast Asia region.

5.3 Use of sequence data to design improved and tailored molecular assays for Southeast Asian countries

The need for rapid and specific diagnostic method has been highlighted in the SEAFMD 2020 roadmap. In this current study, multi lineages FMDV have been found to be co-circulating in Southeast Asian countries particularly serotype O. In some of laboratories in Southeast Asian countries, real-time PCR machines are readily available and widely used in diagnosis or research. Locally in Malaysia, many laboratories have real-time PCR machines but do not readily have access to machines for sequence analyses. Therefore a real-time RT-PCR assay approach could become a more realistic for diagnosis and lineage characterisation in these laboratories. Through discussion with colleagues from Vietnam, we look at the potential of designing tailored and specific molecular assay to facilitate the current diagnostic methods in place. These assays can be used to specifically diagnose FMD viruses causing infection not only at the serotype level but also to recognise the lineages that are present.

Probe-based primer sets for real-time RT-PCR (TaqMan® format) for five lineages (O/SEA/Mya-98, O/ME-SA/PanAsia, O/CATHAY, A/ASIA and Asia 1) were designed. These lineage specific primer sets were designed to target the VP1 gene focussing on specific motifs of each lineage. The VP1 sequences; 95, 50, 18, 107 and 47 sequences for each lineages; O/SEA/Mya-98, O/ME-SA/PanAsia, O/CATHAY, A/ASIA and Asia 1 respectively were aligned together in BioEdit sequence alignment editor v.7.0.5.3. Areas of conserved motifs between sequences within each lineage (sensitivity) were identified also with consideration of inter-lineages differences (specificity). A representative sequence for each lineage was

chosen and oligonucleotides for forward primer, probe and reverse primer were designed using Primer Express™ v1.0 (Applied Biosystems, UK). The target motifs for the primers were compared between lineages and in-silico analysis was carried out to determine the putative sensitivity and specificity of these primer sets.

The sensitivity and specificity of the primers designed in this study are summarised in graphs presented in **Figure 5.2a-5.2e**. These preliminary data indicate that there is possibility to design lineage specific primers for some lineages especially serotypes A and Asia 1 which show high percentage identity between sequences within each of the lineages but are highly variable with the other lineages across the region targeted by the assay. In contrast, to design spesific primers and probe for serotype O lineages is more challenging. Nucleotides are observed to be conserved between lineages in this serotype and it is quite difficult to identify conserved motifs that are unique to these lineages. This is obvious when primers for PanAsia and Mya-98 were compared (**Figure 5.2c and 5.2d**).

This preliminary study designed primers that are intended for TaqMan® real-time analyses. This assay format has been established for pan-serotypic FMDV detection targeting the 5' UTR and 3D region (Callahan et al., 2002; Reid et al., 2002). The availability of lineage specific primers will hopefully enhance the usage of real-time RT-PCR assay in FMD research and disease surveillance. Besides TaqMan® real-time assays, other assay formats have also been developed for FMDV. Loop-mediated isothermal amplification (LAMP) assay for FMDV detection has shown to be useful for rapid FMD diagnosis without the need of sophisticated instrumentation (Dukes et al., 2006). In future, with an advance assay of lineages specific FMDV detection by real-time RT-PCR, it could be possible to extend the assay application for LAMP.

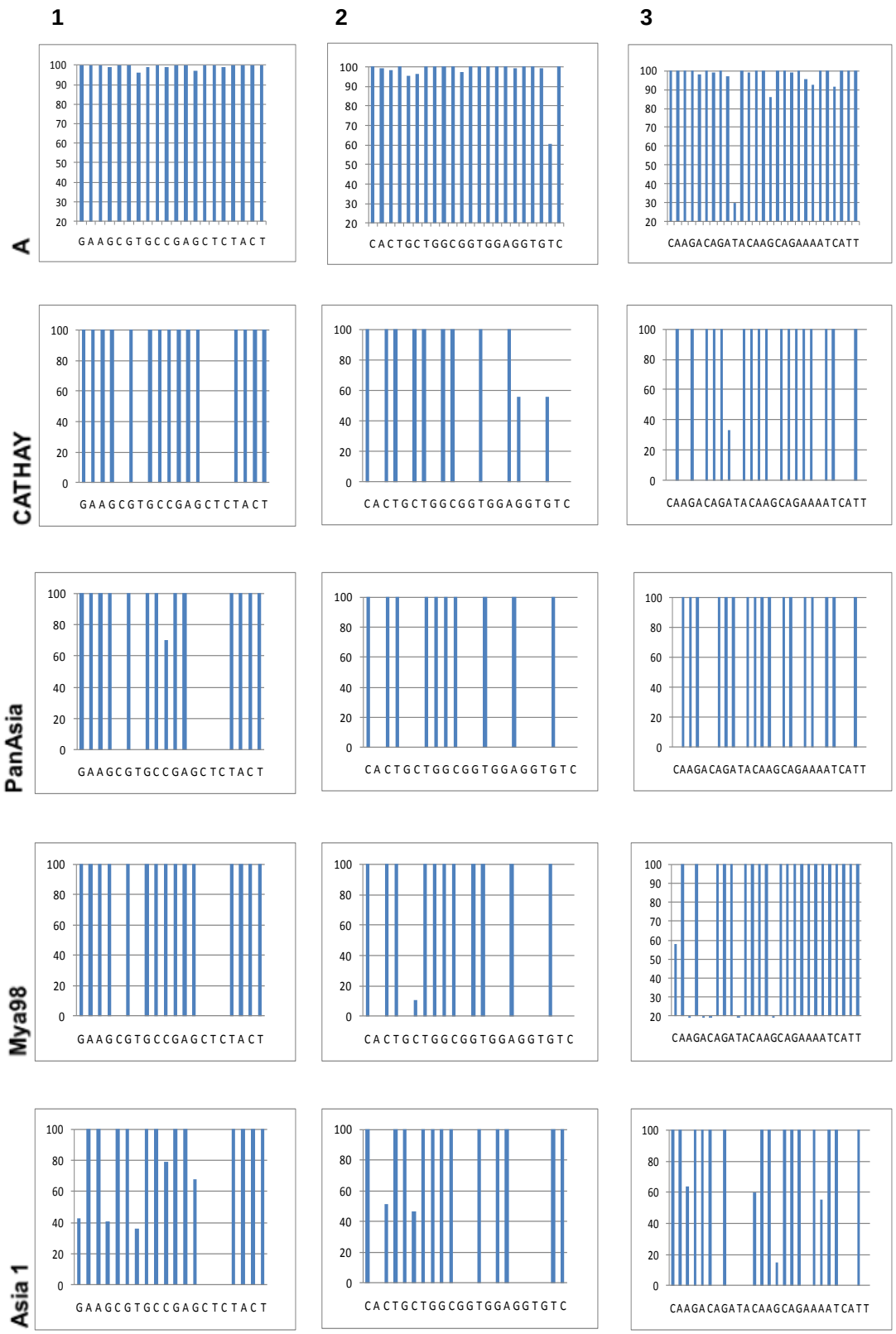
1
2
3
A
CATHAY
PanAsia
Mya98
Asia 1
GAAGCGTGCCGAGCTCTACT
CACTGCTGGCGGTGGAGGTGTC
CAAGACAGATACAAGCAGAAAATCATT

Figure 5.2a

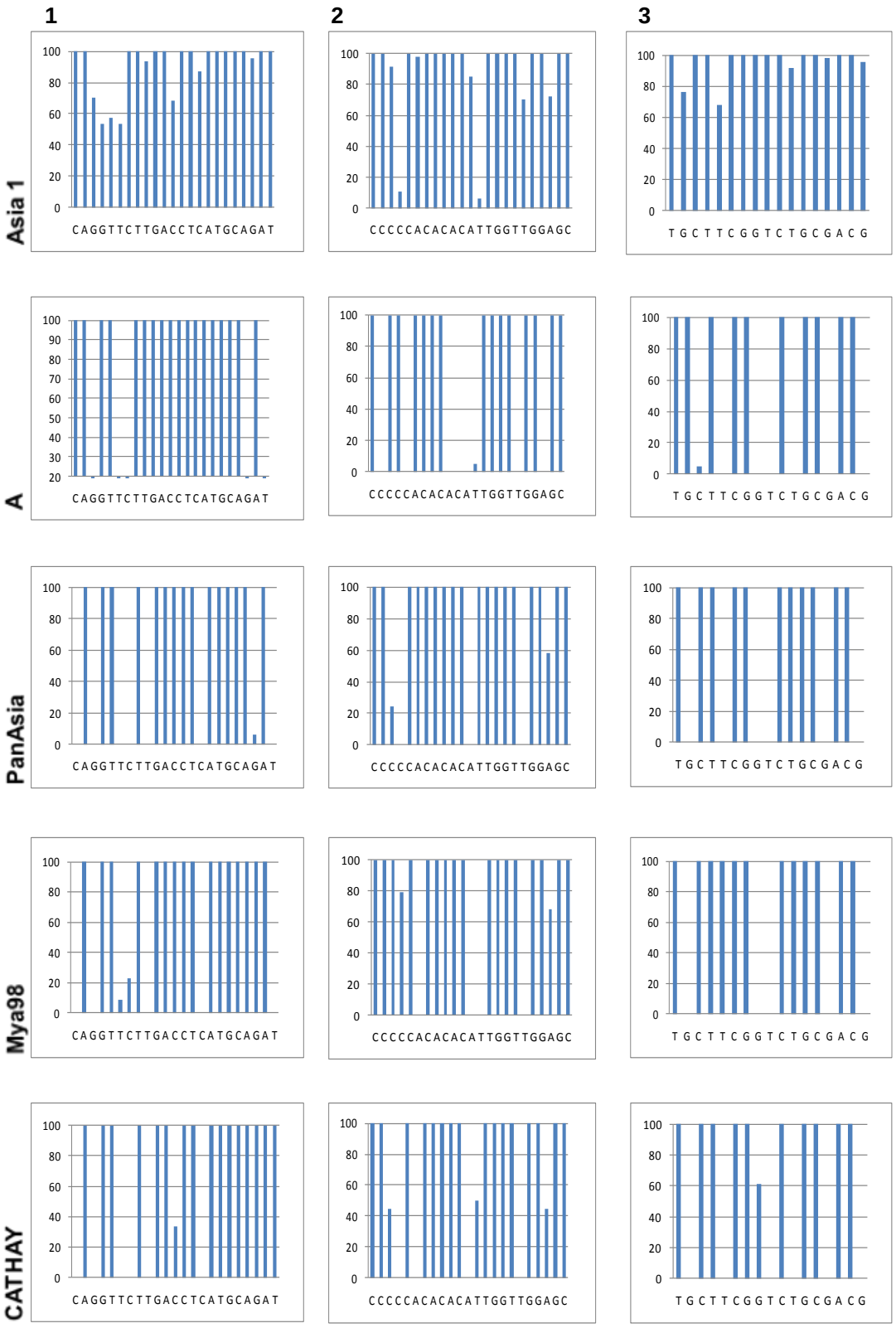
1
2
3
Asia 1
A
PanAsia
Mya98
CATHAY
CAGGTTCTTGACCTCATGCAGAT
CCCCCACACACATTGGTTGGAGC
TGCTTCGGTCTGCGACG

Figure 5.2b

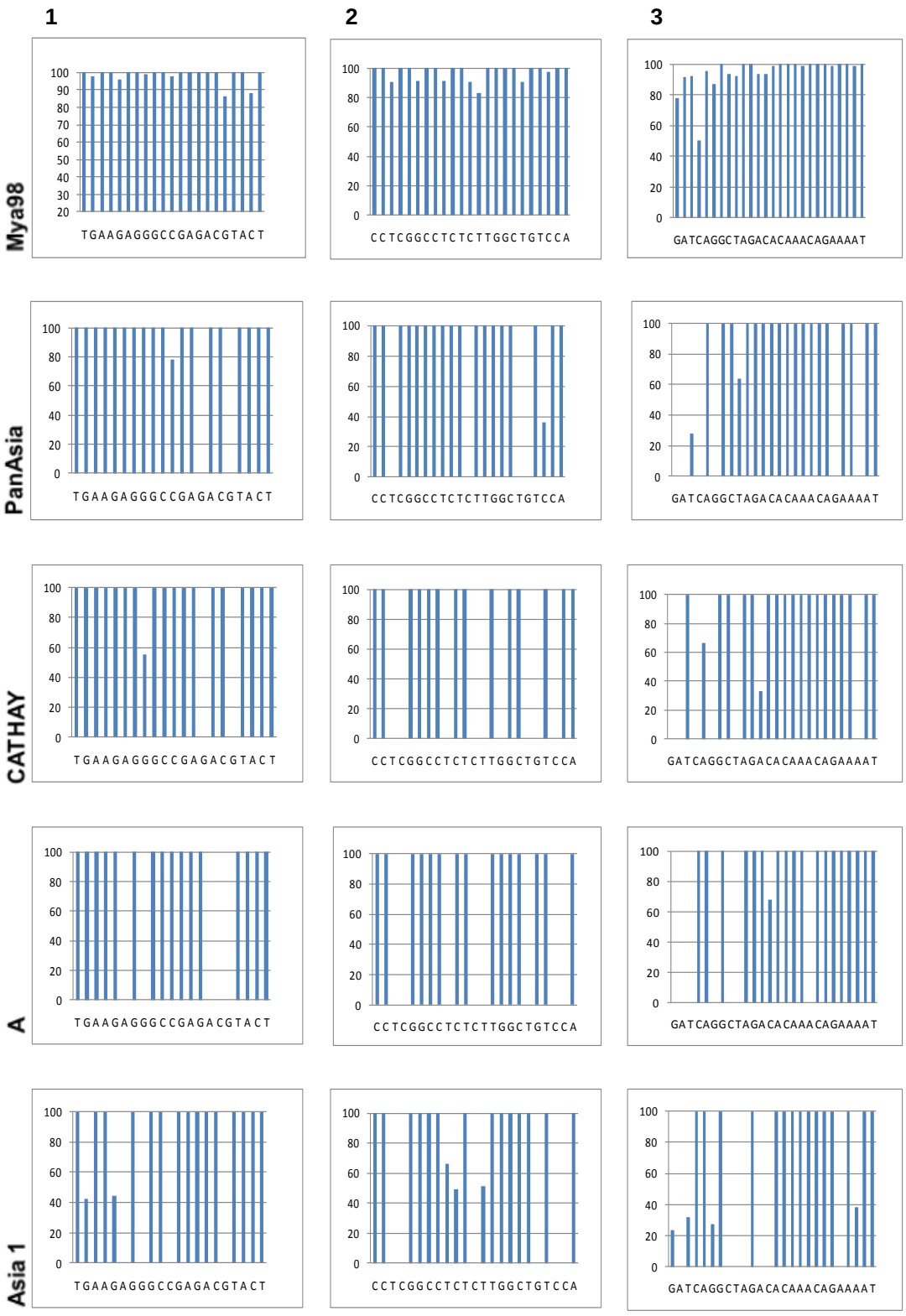
1
2
3
Mya98
PanAsia
CATHAY
A
Asia 1
TGAAGAGGGCCGAGACGTACT
CCTCGGCCTCTCTTGGCTGTCCA
GATCAGGCTAGACACAAACAGAAAAT

Figure 5.2c

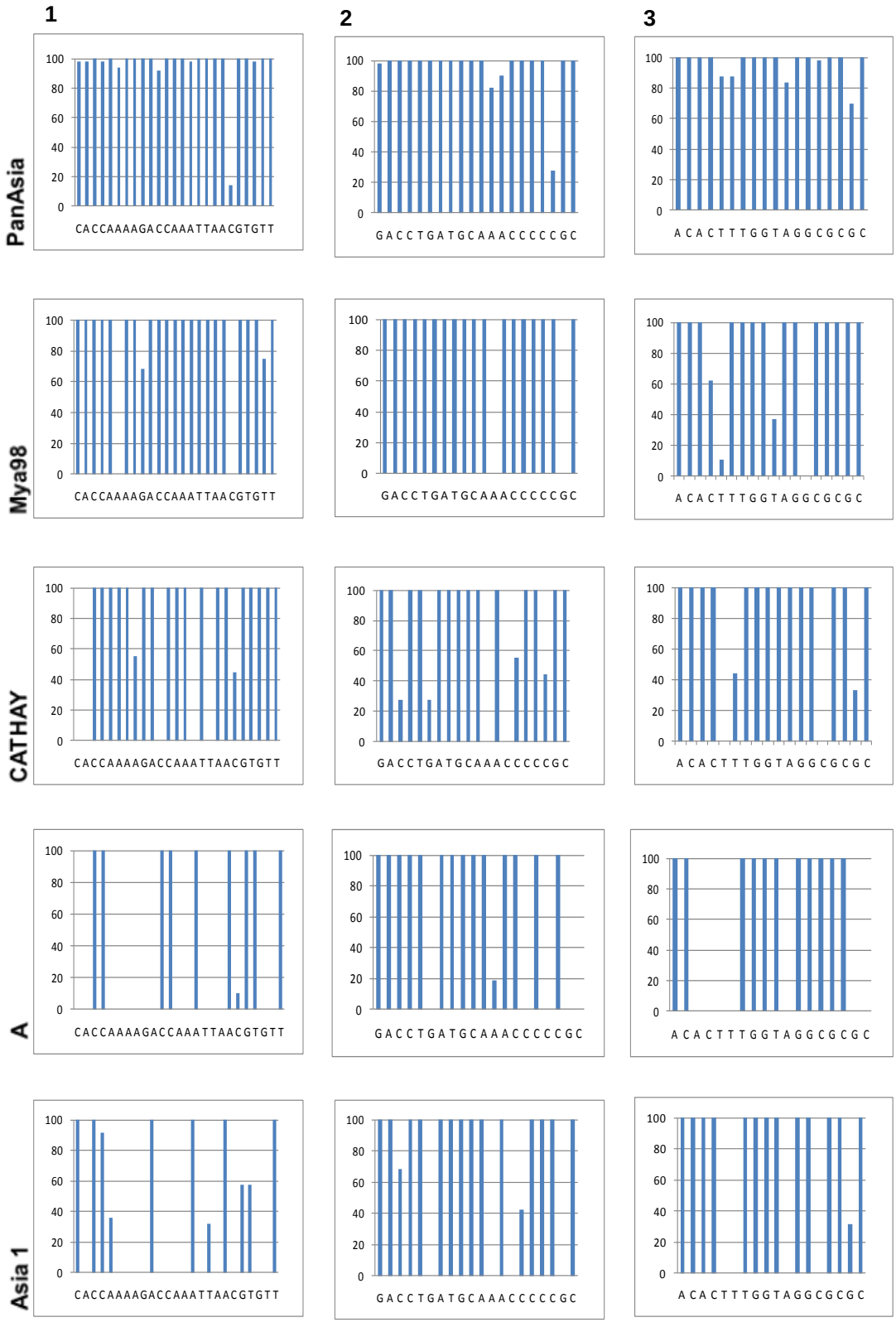
1
2
3
PanAsia
Mya98
CATHAY
A
Asia 1
CACCAAAAGACCAAATTAACGTGTT
GACCTGATGCAAACCCCCGC
ACACTTTGGTAGGCGCGC

Figure 5.2d

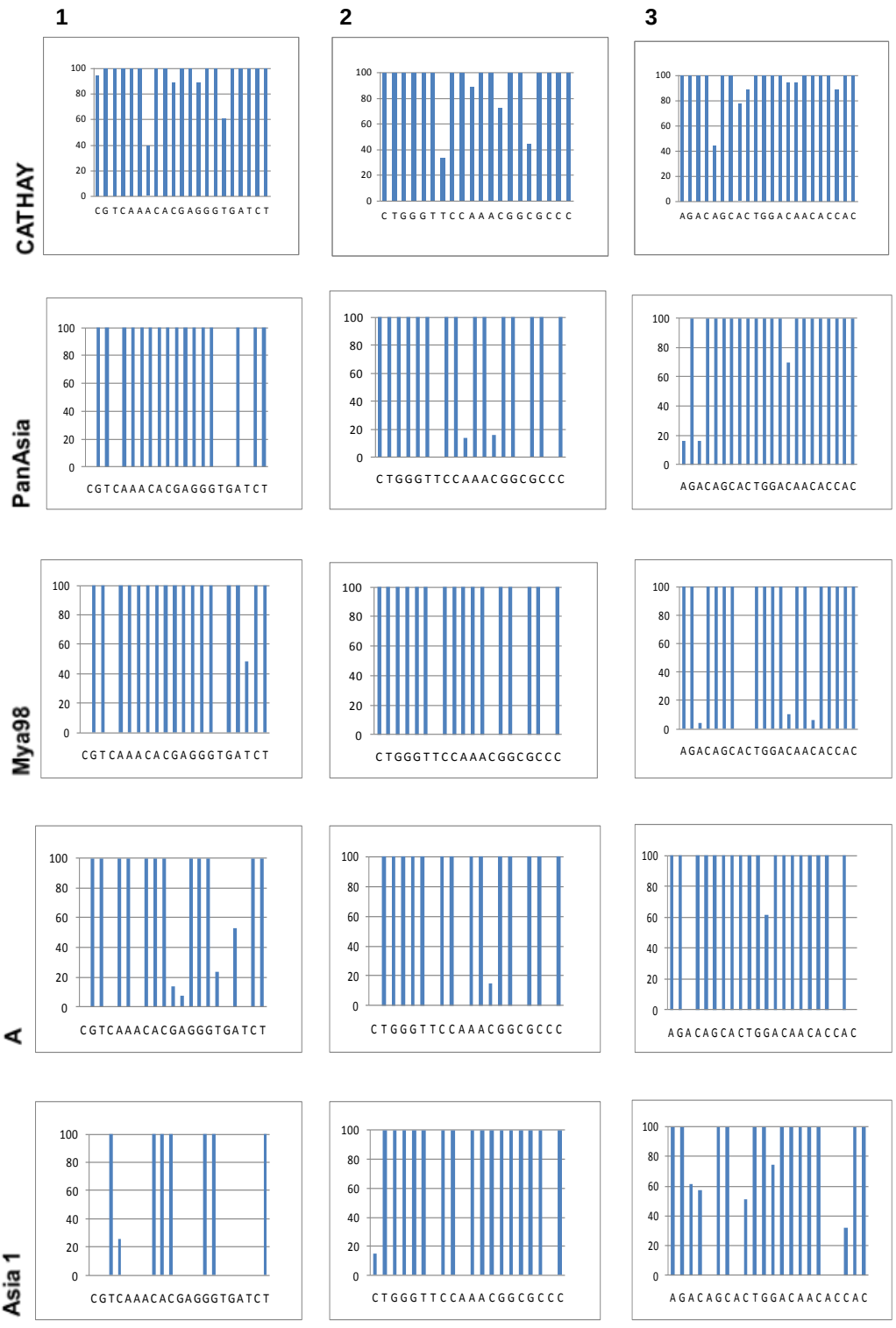

Figure 5.2e

Figure 5.2a-e: Graphs showing in-silico analyses of percentage nucleotide identities (y-axis) of represent real-time RT-PCR oligonucleotides for (a) serotype A, (b) serotype Asia 1, (c) O/SEA/Mya-98, (d) O/ME-SA/PanAsia and (e) O/CATHAY. The first rows are intra-lineage nucleotide identities for the forward primer (1), probe (2) and reverse primer (3) respectively (sequences of each respective oligonucleotide are shown on the x-axis). Subsequent rows show inter-lineage nucleotide identities for these assays.

5.4 Future challenges

Southeast Asian countries can be considered as fast growing economic countries compared to other developing countries resulting in rapid changes in a population growth and demographics as well as increased in trade and tourism. These factors contribute to the increased rate of transboundary diseases such as FMD due to animal or animal product movements either legally or illegally crossing international boundaries to meet the increasing demand. A regional disease control program called SEAFMD was initiated in 1997 in Southeast Asia targeted to eradicate FMD through progressive zoning with vaccination by 2020. The long term strategy was outlined in SEAFMD road map 2020 (details available at http://www.seafmd-rcu.oie.int/documents/SEAFMD%202020%20WEB%20Version.pdf). The SEAFMD campaign provides a network to connect each of the member countries; Malaysia, Thailand, Myanmar, Lao PDR, Cambodia, Vietnam, Indonesia and Philippines.

The first three phase of the campaign (1997-2010) have shown a considerable achievement including the establishment of networks between countries, continuous training, improved laboratory infrastructure, strengthened surveillance and disease management systems as well as enhanced public awareness and communication. However, two phases (phase 4 and 5) that lie ahead generate further challenges for the SEAFMD campaign. In the 16th Meeting of the OIE Sub-Commission for the FMD in Southeast Asia in Vientiane Loa PDR (15-19 March 2010), the increasing number of FMD outbreaks in Southeast Asia countries has been highlighted and the challenges have been defined. The most important strategy was to strike FMD at its source in order to break the infection cycle and prevent it from spreading. Apart from that, other elements that were also given attention were; a review on

appropriate vaccines for use in a vaccination program; improved understanding of the molecular epidemiology of FMD outbreaks by enhanced sample collection and analysis; strategic intervention at critical points in the movements of animals in the region and enhance roles for laboratories in diagnostic and surveillance analysis.

The other challenges to be faced by Southeast Asian countries are the fact that the neighbouring countries in the northern part (People's Republic of China) and western part (Indian sub-continent) are endemic with the disease. This may hamper the disease control effort in place and poses a continuous threat of virus introduction from these neighbouring regions. A similar model of disease control program via progressive zoning has been adapted by Southern America countries. The advantage of the region is they are neighbouring to Northern America which is FMD-free (without vaccination) and therefore do not pose a risk of virus re-introduction. Therefore the threat of FMDV introduction into Southern American countries is low and subsequently contributes to the effectiveness of disease control program.

Generally, countries in Southeast Asia are poor to moderate affluent countries where animal farming and husbandry are practised usually on a small scale (small holdings). In these countries, farmer's views towards FMD are different compared to farmers in FMD-free countries. FMD is often viewed as a non-life threatening disease, therefore there's no urgency to treat or to involve any local authority. There is therefore a critical need to educate animal owners about the importance of this disease to them, and an understanding of the benefits they would get if FMD was eradicated from their herd.

The important thing that these endemic countries have to learn from those FMD-free countries is incentives for cooperating with national authorities to fight this disease. Infrastructure has always become a problem especially to ensure that

sufficient vaccines are deployed in suitable conditions. Furthermore, the lack of controls at the borders resulted in uncontrolled movements crossing international borders. As long as these general challenges have not been overcome, the effort to control FMD in the region will definitely be a difficult task.

This study provides proof that sophisticated molecular assay is very much needed for FMD diagnosis in Malaysia. The initial step after finishing this study is to transfer the knowledge especially the VP1 sequencing method locally in diagnostic laboratory in Malaysia. This method is quite easy but a sophisticated instrument such as sequencing machine is needed which can be out sourced in private lab in Malaysia. This would definitely pose a challenge due to amount of money needed but in order to meet the SEAFMD campaign, it must be done. I believe that Malaysian government is committed to the achievement of FMD-free status by the year 2020. The complete genome sequences assays hopefully will enhance the capability of molecular analysis in WRLFMD especially when dealing from FMDV from the Southeast Asian countries and hopefully one day these protocols can be employed locally.

In conclusion, we hope the tools presented and generated in this thesis will be a small step in generating the resources needed to reduce and eventually eradicate this important disease from Southeast Asia.

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Appendixes

Appendix 1: RNA extraction from virus using QIAGEN RNeasy® Mini Kit (Cat. No. 74104)

1. Read carefully the instruction manual provided by the manufacturer.
2. In a Class 2 laminar flow cabinet, put 460µl of virus sample into a 1.5 ml tube.
3. Add an equal volume of Lysis buffer RLT containing 1% 2 mercaptoethanol (SIGMA) to the sample.
4. Mix using a vortex mixer (set the vortex mixer to constant rapid rotation and touch the tube against the rubber cup for 5-10 sec).
5. Add 460µl 70% ethanol and mix using a vortex mixer.
6. Apply to RNeasy spin column (700 µl maximum loading volume).
7. Spin in a microfuge for 10-15sec at 10,000-12,000rpm (7,000-10,000g).
8. Discard flow-through and reuse collection tube.
9. Repeat with remaining volume.
10. Wash with 700µl wash buffer RW1. Centrifuge for 10-15sec at 10,000-12,000rpm (7,000-10,000g). Discard flowthrough and reuse collection tube.
11. Wash with 500 µl wash buffer RPE. Centrifuge for 10-15 sec at 10,000-12,000rpm (7,000-10,000g).
12. Discard flowthrough and reuse collection tube.
13. Repeat wash with 500µl wash buffer RPE. Centrifuge at max speed for 2 min to dry membrane.
14. Discard flow-through and collection tube.
15. Transfer column to a new collection tube and centrifuge at max speed for 1 min to remove any traces of ethanol. Discard collection tube. Place column on to a new 1.5 ml eppendorf tube.

16. Elute RNA with 50 µl nuclease-free water into the new 1.5 ml tube.
17. Spin in a microfuge for 60sec at 10,000-12,000rpm (7,000-10,000g).
18. Store in clean tube in freezer between -30°C and -5°C or -50°C and -90°C. Label the tube clearly with “RNA”, the virus name/passage history, the date and your name (initials).

Appendix 2: One-step reverse transcription and polymerase chain reaction (RT-PCR) to amplify viral RNA using QIAGEN® OneStep RT-PCK Kit (Cat. No. 210212)

1. Read carefully the instruction manual provided by the manufacturer.
2. Add the following reagents (in the order shown) into a 0.25 ml thin-walled tube. Take care to use sterile tips to avoid cross contamination. Use new tips for each reagent and RNA. One positive control (positive RNA for FMDV and for the serotype search by RT-PCR) and NF (nuclease-free) water as negative control are included.

   Table 1: RT-PCR reaction mixture:

Component	Volume for 1 reaction
RNase-free water	16.0 µl
5x QIAGEN OneStep RT-PCR buffer (containing 12.5mM $MgCl_2$)	10.0 µl
dNTP mix (containing 10mM of each dNTP)	2.0 µl
Forward primer (4pmol/µl)	5.0 µl
Reverse primer (4pmol/µl)	10.0 µl
QIAGEN OneStep RT-PCR enzyme mix	2.0 µl
Template RNA	5.0 µl

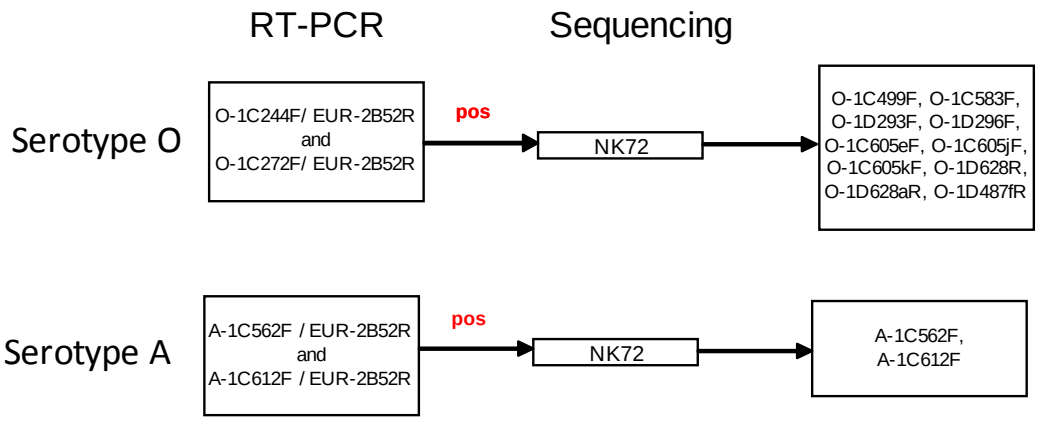
3. Place tubes in thermocycler and select relevant cycling program for each FMDV serotype as in Table 2. The thermocycler should be set to hold the tubes at 4°C after the cycling program has finished.

Table 2: RT-PCR thermal condition

Protocol	Temperature	Time	No. of cycles
FMDV-A	50 °C	30 min	1
	95 °C	15 min	1
	95 °C	60 sec	35
	55 °C	60 sec	
	72 °C	120 sec	
	72 °C	10 min	1
FMDV-O	50 °C	30 min	1
	95 °C	15 min	1
	95 °C	60 sec	35
	60 °C	60 sec	
	72 °C	120 sec	
	72 °C	10 min	1

4. The decision to use oligonucleotide for RT-PCR and sequencing are illustrated in Figure 1. Refer to Supplement Table 3 for oligonucleotide sequences.

Figure 1: Decision tree for RT-PCR and sequencing.



5. Primers for RT-PCR are tested simultaneously while sequencing primers are chosen based on NK72 sequences.

Appendix 3: Two step RT-PCR

Reverse transcriptase (RT) using Superscript™ III Reverse Transcriptase kit (Invitrogen- Cat. No. 18080-093)

1. To prepare cDNA, add 3 µl 10 µM UKFMD Rev 6 primer (GGC GGC CGC TTT TTT TTT TTT TTT) , 3 µl 10mM dNTP and 15 µl RNA in 0.2 ml eppendorf tube. Do this in 3x reaction (3x15 µl) to obtain 120 µl cDNA

2. Place in thermocycler at 70°C for 3 minutes and 4°C for 3 minutes.

3. Prepare RT mix [single reaction consist of 5x FS buffer (8 µl), 0.1mM DTT (2 µl), RNase OUT (2 µl) (Invitrogen, Cat. No. 10777-019), Superscript III RT (2 µl) and NF water (5 µl)].

4. Add the master mix (19 µl) into tube from step 2.

5. Place in thermocycler at condition of 45°C for 60 minutes, 85°C for 5 minutes and hold at 4°C.

6. Clean up the RT product using GFX column as instructed by the manufacturer and detailed in Appendix 5. Pool cDNA from 3 reactions to obtain a total of 120 µl.

PCR using Platinum® *Taq* DNA Polymerase High Fidelity kit (Invitrogen- Cat. No. 11304-011)

1. In a clean room set up required PCR mix for required sets of primer with negative control for each reaction.

2. The PCR mix for one reaction consist of 10x PCR buffer (5 µl), 50mM $MgSO_4$ (2 µl), 10mM DNTP (1 µl), Platinum® High Fidelity Taq (0.25 µl) and NF water (34.76 µl).

3. Add 43 µl master mix into each tube and add 2 µl of each 10 µM forward primer and 10 µM reverse primer.

4. In PCR room add 3 µl cDNA to give final volume of 50 µl.

5. Place tubes in thermocycler. Run the PCR program cycle of initial denaturation at 94°C for 5 minute, then 39 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, finishing the cycle with incubation at 72°C for 5 minutes.

6. Run the 1.5% agarose gel to check the PCR product at 100V for 40 minutes alongside a 100 bp ladder.

Appendix 4: Cycle sequencing reaction using BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI- Cat No. 4337455)

1. Clean the PCR product or cDNA using the GFX column as explained in Appendix 5.

2. In clean room set up plate for cycle sequencing reaction depending on number of PCR reaction subjected for sequencing. For each PCR product there will be two sequencing reactions. One for forward primer and one for reverse primer.

3. Each reaction using 7.5 µl of master mix which contain 0.25 µl BigDye® Terminator, 1.88 µl 5x sequencing buffer and 5.37 µl NF water.

4. Add 1.5 µl of 1.6 µM* primer for each reaction.

   *to make 100 of 1.6 µM: add 16 µl of 10 µM primer into 84 µl NF water.

5. Add 1 µl template DNA to give final volume of 10 µl.

6. Run the plate on a program of 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds and 50°C for 5 seconds, finishing the cycle with incubation at 60°C for 4 minutes.

7. Clean up the reaction by ethanol precipitation (Appendix 6).

Appendix 5: PCR purification protocol using GFX™ PCR DNA and Gel Band purification Kit (Cat. No. 28-9034-70)

1. Read the manual instruction provided by the manufacturer carefully.
2. Place a GFX spin column in a provided 2 ml collection tube.
3. Add 500 µl of capture buffer type 3 on to the column and followed by all the PCR product.
4. Spin the tube at 12,000 rpm for 1 minute.
5. Discard flow-through. Place the GFX column back into the same tube.
6. Add 500 µl wash buffer type 1 to the GFX column and centrifuge at 12,000 rpm for 1 minute.
7. Place GFX column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 40 µl elution buffer type 6 for sequencing purpose or type 4 for cDNA clean up. Apply the elution buffer to the centre of the GFX membrane, let the column stand for 1 min, and then centrifuge the column at 12,000 rpm for 1 min.

Appendix 6: Ethanol and EDTA clean up

1. Add 5 µl 125 mM* EDTA (0.5M, Sigma, Cat. No: E7889) and 60 µl 100% ethanol to the 10 µl sequencing reaction.

   * do 1 in 4 dilutions to obtain 125mM EDTA from 0.5M EDTA.
2. Mix the tube or plate with vortex for 15 seconds and leave in the dark at room temperature for a minimum of 15 minutes
3. Centrifuge at 2000xg (approximately 2500 rpm) for 45 minutes.
4. Discard the supernatant carefully by removing the cap or adhesive tapes and invert the plate onto a paper towel. Place the plate upside down on a paper towel and quick spin (not more than 100 rpm) to ensure all supernatant was removed.
5. Add 60 µl of 70% ethanol and centrifuge at 1000xg (approximately 1650 rpm) for 15 minutes.
6. Repeat step 4 to discard the supernatant.
7. Dry the pellet in a vacuum drier for about 30 minutes or until no ethanol excess was present.
8. Resuspend the pellet with 20 µl Hi-Di™ Formamide (ABI, Cat No. 4311320) and leave in the dark at room temperature for minimum 15 minutes. If sequencing is not carried out immediately, pellet can be kept in -20ºC for longer storage.

9. Run the plate in sequencing machine (3730 DNA Analyzer, Hitachi, Applied Biosystems).